An ultrastructural study of gametogenesis and early development in the sea anemone *Actinia fragecea* (Cnidaria : Anthozoa)

Thesis

An Ultrastructural Study of Gametogenesis and Early Development in the Sea Anemone *Actinia fragacea* (Cnidaria: Anthozoa).

Alan Urquhart LARKMAN

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Author's number: HDJ 0189
Date of submission: November 1985
Date of award: 5 February 1986

The Open University

Discipline: Biology

November; 1984

PAGE
NUMBERS
CUT OFF
IN
ORIGINAL

STATEMENT

An Ultrastructural Study of Gametogenesis and Early Development in the Sea Anemone *Actinia fragacea* (Cnidaria: Anthozoa).

Alan Urquhart LARKMAN.

I agree that the above work, if approved for the degree of Doctor of Philosophy and deposited in the University Library:

i) may be made available at the discretion of the librarian

ii) may be photocopied at the discretion of the librarian.

. . . .

A.U. Larkman

ABSTRACT

Large individuals of the sea anemone *Actinia fragacea* were collected at approximately monthly intervals over a two year period. Their gonads were examined by light and electron microscopy, in order to follow the gametogenic process. The sexes are separate, and both show an annual cycle of activity. Oocytes arise in the gonad epithelia, but soon migrate into the mesogloea. During vitellogenesis, the oocytes accumulate compound yolk granules, fibrillar and cortical granules, lipid droplets and glycogen. The surface of large oocytes bears tufts of large microvilli or cytospines. The oocytes reach some 150 μm in size. A group of specialized gonad epithelial cells projects through the mesogloea and contacts the oocyte surface, forming the trophonema, which is involved with nutrient transfer. The gonad epithelium can take up nutrients from the external medium, and the trophonema is particularly active in the uptake and incorporation of some small molecules. Not all fully grown oocytes are always spawned; some break down in an orderly fashion and are resorbed. Oogenesis was also examined, in less detail, in four other species of anemone. Spermatogenesis takes place in spherical testicular cysts, which are also associated with trophonemata. Spawning occurred in the laboratory on three occasions. Spawned eggs do not possess a vitelline coat, and do not undergo a cortical reaction. Gastrulae may take up numerous supernumary sperm.

ACKNOWLEDGEMENTS

I am indebted to many staff of the Department of Biological Sciences, Portsmouth Polytechnic, for their assistance during the course of this study. Particular mention must be made of a few - Mr. Mick Revill, Chief Technician in Zoology, for his patience and skill in the construction and repair of numerous items of equipment; Mr. Graham Bremer for assistance with scanning electron microscopy; Miss Christine Preston for many helpful discussions, and Lynn Healey for her very considerable assistance in the preparation and typing of the manuscript.

I am also grateful to my internal supervisor at the Open University, Dr. M.G. Stewart, for his willing help and advice, and to the Open University Crowther Fund for financial assistance during the latter stages of the project.

Above all, however, I am grateful to my external supervisor, Dr. M.A. Carter for his constant faith, support and practical help during all stages of the work, but, most memorably, for his unfailing and infectious enthusiasm for so many aspects of biological science.

CONTENTS

VOLUME II

PUBLISHED WORK

Parts of the content of several chapters of this Thesis have been published in the form of papers in scientific journals. These are:-

Chapter 4

Larkman, A.U. (1981). An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria: Anthozoa). International Journal of Invertebrate Reproduction, 4: 147-167.

Chapter 5

Larkman, A.U. (1983). An ultrastructural study of oocyte growth within the endoderm and entry into the mesogloea in *Actinia fragacea* (Cnidaria: Anthozoa). Journal of Morphology, 178: 155-177.

Chapter 6, Section C

Larkman, A.U. (1984). The fine structure of mitochondria and the mitochondrial cloud during oogenesis in the sea anemone *Actinia*. Tissue and Cell, 16: 393-404.

Chapter 6, Section I

Larkman, A.U. (1984). The fine structure of granular amoebocytes from the gonads of the sea anemone *Actinia fragacea* (Cnidaria: Anthozoa). Protoplasma, 122: 203-221.

Chapter 7

Larkman, A.U. and Carter, M.A. (1982). Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a

α

nutritive function. International Journal of Invertebrate Reproduction, 4: 375-379.

Chapter 11

Larkman, A.U. (1984). An ultrastructural study of the establishment of the testicular cysts during spermatogenesis in the sea anemone *Actinia fragacea* (Cnidaria: Anthozoa). Gamete Research, 9: 303-327.

Chapter 13

Larkman, A.U. and Carter M.A. (1984). The apparent absence of a cortical reaction after fertilization in a sea anemone. Tissue and Cell, 16: 125-130.

Reprints or photocopies of these publications are included inside the back cover of this volume. Two other publications, relevant to this study, but describing work carried out wholly or partially prior to the commencement of the period of Degree Registration, have also been included. They are:-

Larkman, A.U. and Carter, M.A. (1980) The spermatozoon of *Actinia equina* L. var *mesembryanthemum*. Journal of the Marine Biological Association of the United Kingdom, 60: 193-204.

Larkman, A.U. (1980) Ultrastructural aspects of gametogenesis in *Actinia equina* L. in P. Tardent and R.Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North Holland, pp. 61-66.

Chapter 1

GENERAL INTRODUCTION

Interest in the means by which new animal and human individuals develop and are brought into being extends back to the earliest phases of human history. Shapiro and Eddy (1980) mention evidence of cave paintings and artefacts from neolithic times indicating the fascination shown by early man for the generative process. Ancient Egyptians speculated upon the point at which the developing embryo became animated by the deity, and also perfected a highly successful technique for the artificial incubation of birds' eggs (Needham, 1931). Serious intellectual study and debate of embryological matters was pursued in ancient Greece, as is testified by the surviving writings of Hippocrates and Aristotle. With the dawn of experimental science, natural curiosity and the quest for understanding of reproductive processes have been sharpened and reinforced by the desire to manipulate these processes, in order to reduce or enhance the fecundity of man and his domestic animals. Nevertheless, in 1931, Needham wrote that a line "as straight as Watling Street" ran from Hippocrates through Aristotle, Leonardo, Harvey and von Baer to the current issues of developmental biology journals.

During the last 30 years or so, with the development of the electron microscope and the rapid expansion of the new science of cell biology, the study of gametogenesis has received a further impetus. Animal eggs and sperm are extreme examples of specialized cell types, and so may serve as model systems for the study of specific cellular activities. For example, developing oocytes are specialized for the uptake or synthesis of reserve materials, and they and their associated

cells can make suitable subjects for the investigation of such processes. In many animal species, the egg is the largest cell produced by the organism, while the sperm is usually among the smallest. However, both are usually derived from cells of only moderate size and often unremarkable appearance. Gametogenesis may thus offer an opportunity to observe a remarkable series of cellular transformations, and probe the mechanisms by which they are achieved.

While the greatest significance in medical and economic terms is always likely to be attached to the study of mammals and other higher animal groups, in gametogenesis and many other areas of biology, our overall understanding owes much to the consideration of many different animal types. Some groups show characteristics which render them particularly convenient for a given type of experimental manipulation. Thus the eggs and embryos of amphibians and birds are generally larger and more easily obtained than those of mammals, and so were the material of choice for experimental embryologists for many years. Eggs and sperm may be obtained very easily from some echinoderm species, and have figured prominently in studies of fertilization and egg activation in recent years. But there may be factors other than convenience which favour the use of organisms phylogenetically far removed from mammals as objects of research. Many advances have stemmed from the investigation of lower organisms whose systems may be less sophisticated and hence more easily analysed than those of higher groups. The advance of molecular biology owes much to the study of the simplest organisms available in the form of bacteria and their viruses. By building on the understanding achieved with these organisms, the more complex problems encountered in higher

organisms can now be approached with some confidence. The study of simpler organisms may also give clues to the paths followed during the evolution of more complex mechanisms.

Coelenterates are among the most primitive of all multicellular organisms. Perhaps because of some of the factors outlined above, interest in many areas of their biology has increased in the last 20 years or so. Much of this attention has been focussed on the freshwater polyps of the genus *Hydra*. Workers using *Hydra* species have made a long and distinguished contribution to biological research, dating back to the classical studies of Abraham Trembley (1710-1784), reviewed in an interesting recent account by Lenhoff (1980). In many respects, *Hydra* has proved to be a remarkably convenient experimental animal. It may be cultured relatively easily in enormous numbers in the laboratory (Lenhoff and Brown, 1970). It has great regenerative powers, and pieces of *Hydra* can easily be grafted together, enabling sophisticated experiments on the mechanism of pattern formation to be carried out (e.g Wolpert, 1969). Its simple body plan has enabled models for the control of morphogenesis to be devised, and, to some extent, tested experimentally (Gierer, 1977; Gierer and Meinhardt, 1972). Its cellular composition and dynamics can be analyzed by quantitative methods in a way which has not yet proved possible for any other organism (David, 1973; Bode and David, 1978; David, 1983). In laboratory culture, most species and strains of *Hydra* reproduce only by asexual budding. This has considerable advantages for many investigations, enabling experiments to be carried out using clones of genetically identical individuals. However, *Hydra* is a less than convenient organism for studies of sexual reproduction.

Most *Hydra* can be induced to reproduce sexually only inconsistently, and, the process appears unusual in several respects. Often only a single egg is produced at a time, and the resultant embryo may serve as a resting stage for the survival of adverse environmental conditions. Thus *Hydra* is a less than ideal model, and our understanding of sexual reproduction in coelenterates as a whole has not advanced as rapidly as some other fields. As expressed by Campbell (1974a) "sexual reproduction and embryogenesis, the classic starting point of developmental studies, has never been a primary focal point in cnidarian research."

Some other coelenterates may be more suitable than *Hydra* for research into sexual reproduction, and yet retain its advantages in terms of possible simplicity and primitiveness. Sea anemones are solitary marine polyps of the class Anthozoa which inhabit shallow and coastal waters throughout the world. Most are much larger than *Hydra*, and Spaulding (1974) suggested that gametogenesis in sea anemones offered an opportunity for detailed descriptive and comparative work using both the light and electron microsopes. He went on to say that material should be easily available since many anemones possess developing and mature gonads for long periods each year. In spite of their suitability, however, studies of anthozoan gametogenesis have not been numerous. Roosen-Runge (1977) wrote "Spermatogenesis in Anthozoa needs much more thorough investigation, particularly with the electron microscope." More recently, in a review of coelenterate oogenesis, Beams and Kessel (1983) reported that no ultrastructural account of oocyte development or vitello-genesis in an anthozoan had yet been published. While the last statement is perhaps not strictly accurate, it reflects

the paucity of detailed information available for this group. Thus sea anemones were in a position of some potential usefulness which had not yet been exploited, and this was the state of affairs which led to the present study being undertaken.

In the ten years or so prior to the commencement of the present work, several investigations of gametogenesis in sea anemones had been carried out, principally employing the techniques of wax histology and light microscopy. In the most detailed studies (Chia and Rostron, 1970; Dunn, 1975; Jennison, 1979), the authors examined gonads at regular intervals over an extended period of time, so that all stages of the gametogenic cycle could be observed. Dunn (1975) expressed the view that large samples of anemones, collected at various times of year, must be examined histologically before the sexual character of a species could be ascertained. However, these workers were hampered by the limitations of the light microscope and conventional preparative procedures. Most of the electron microscope studies published (e.g Clark and Dewel, 1974; Dewel and Clark, 1974) dealt with only narrow aspects of oocyte growth or sperm development, and only a fragmentary picture had emerged. The only area to have been examined at all thoroughly at the electron microscope level was the structure of the mature sperm (Dewel and Clark, 1972; Hinsch and Clark, 1973; Hinsch, 1974; Lyke and Robson, 1975; Schmidt and Zissler, 1979). The present study was conceived primarily as an attempt to combine the advantages of repeated, regular sampling of gonads with the vastly superior resolution of detail available by the use of electron microscopy. Thus it was hoped that all stages of gamete development could be examined in some depth, without losing sight of the overall pattern.

The approach would be to take regular (approximately monthly) samples of gonads from a single species of anemone from a restricted locality over a period of two years or so. The gonads would be examined by electron microscopy, but since the quantity of gonad from each sample which could be treated in this way would be small, a parallel light microscope study would also be carried out. This should permit a broader overview of gamete development, and also provide a check that the EM samples were representative. In an attempt to improve preservation and make the best use of the limited resolution available with the light microscope, EM-derived fixation procedures would be employed, and semithin (2 μm) sections would be prepared by modern plastic resin embedding techniques rather than conventional wax histology. For comparative purposes, a small number of other anemone species would be studied in a similar way, but in rather less detail and with longer sampling intervals. If spawning and early development could be followed in any species, it would be approached only with a view to elucidating the function of structures observed during gametogenesis. An overall study of embryonic and larval development might represent too large an undertaking in addition to the gametogenesis programme.

The very common and widely distributed beadlet anemone *Actinia equina* var. *mesembryanthemum*, at first sight seemed an obvious candidate for detailed study. However, previous work, largely from Portsmouth, indicated that:-

a) Only a relatively small proportion (ca. 25%) of even large individuals of this variety possessed gonads at any given time. This would exaggerate the difficulties of a regular sampling programme, and require large numbers of

anemones to be collected from the field.

b) Reproduction in this variety appeared unusual in several respects. Adult anemones of both sexes and even those without apparent gonads brood young in their gastrovascular cavities. The brooded young appear to be genetically identical to their parents, and so are unlikely to have been produced by a conventional sexual process (Cain, 1974; Gashout and Ormond, 1979; Carter and Thorp, 1979; Orr *et al*, 1982).

The other British variety of this species, *A. equina* var. *fragacea* does not brood, however, and a very high proportion of large individuals contain recognizable gonads. It was thus decided to study this variety in detail, and use the var. *mesembryanthemum* as one of the anemones selected for less detailed study.

During the early part of the present study, a paper was published (Carter and Thorpe, 1981) suggesting that the ecological, genetic and reproductive differences between these two varieties were such that they should be considered as separate species. This had been suggested independently by a previous report (Rostron and Rostron, 1978). Carter and Thorpe (1981) proposed that the two species be known as *Actinia equina* L. and *Actinia fragacea* (Tugwell, 1856), and their nomenclature has been adopted during the subsequent writing of this thesis. *Actinia fragacea* is less widely distributed in Britain than *A. equina*, but is found on rocky shores around the south west of England. It is oviparous, and tends to occur lower down on the shore than *A. equina*.

The anemones selected for less detailed study were as follows:

1) *Actinia equina*, as described above.

2) *Tealia felina*, a non-brooding anemone found in the same location as *A. fragacea*.

3) *Anemonia sulcata*, another non-brooding intertidal anemone found in the same location, but containing symbiotic zooxanthellae. Unlike the other species studied, it can also reproduce asexually by longitudinal fission.

4) *Cereus pedunculatus*. This is an acontiate or mesomyarian anemone, and so is taxonomically slightly removed from the other species mentioned, which are all endomyarian. It also occupies very different habitats. The population sampled for this study was situated on the intertidal mud flats in the upper reaches of a large sheltered harbour. *Cereus* adults brood young, and their cells contain zooxanthellae.

It was hoped that the above species would provide a range of life-styles and reproductive strategies between which useful comparisons could be made. In fact, there are many similarities in the eggs and sperm of all the species studied, and only major points of difference are described in detail in this thesis.

i) Sea anemone structure

Only the briefest account of anemone structure will be given here, primarily to introduce some of the terms used later in the thesis. A more complete and detailed account is given by Stephenson (1928) among others. Like *Hydra* and all polyps, the anemone body plan is that of a hollow cylinder closed by basal and oral discs. The basal disc is complete, but the oral disc is perforated by a mouth and is fringed by hollow tentacles. The central cavity is known as the coelenteron or

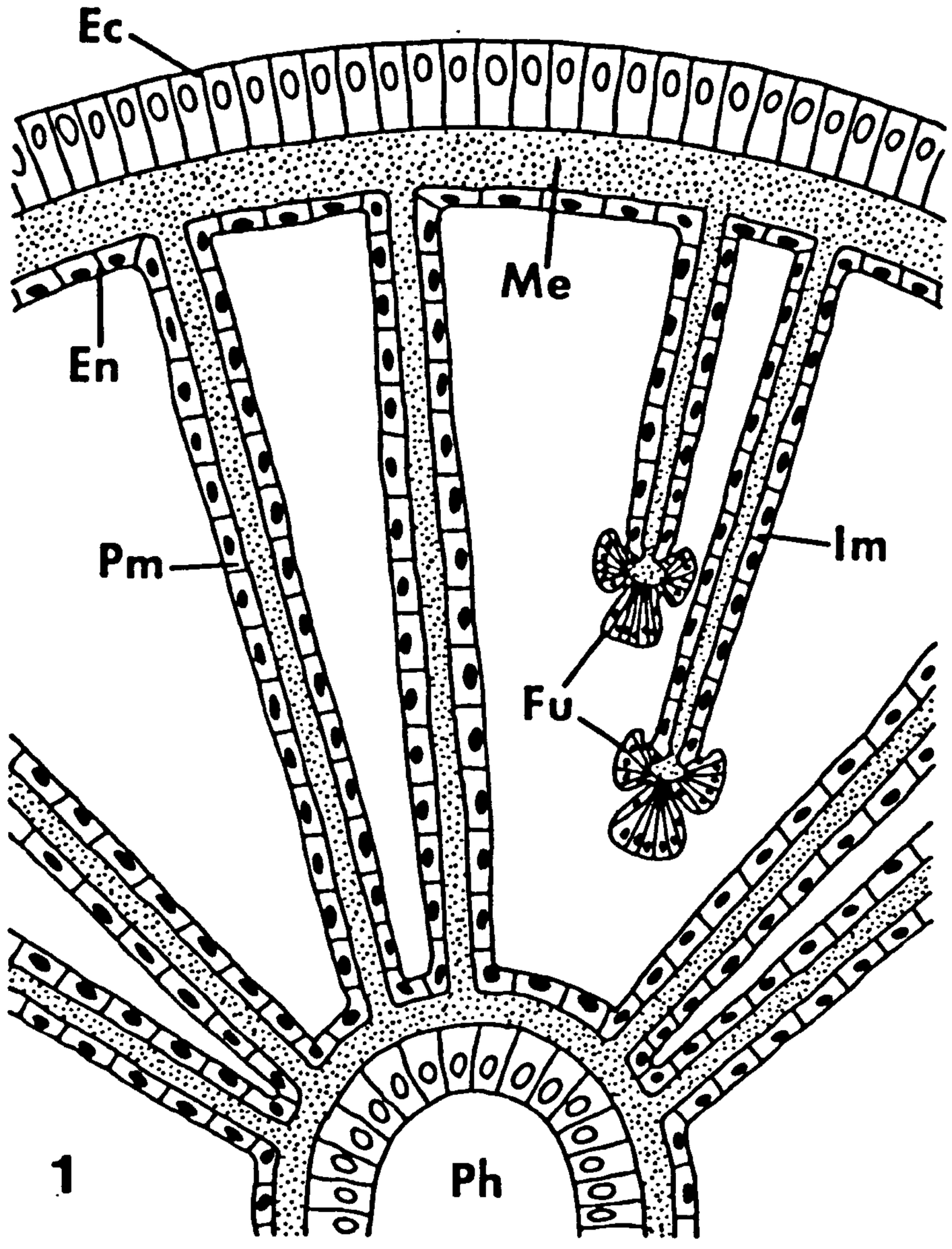
gastrovascular cavity. Anemones differ from *Hydra* in at least two important respects. The mouth does not open directly into the gastrovascular cavity, but rather into a tube known as the pharynx or actinopharynx. Also the gastrovascular cavity is partially divided by radially arranged longitudinal partitions known as mesenteries.

The body wall of all coelenterates consists of three layers, known as ectoderm, mesogloea and endoderm. The ectoderm and endoderm are made up of columnar epithelial cells of several types. In contrast to *Hydra* and other Hydrozoa, where the mesogloea is thin and non-cellular, in anemones it may be thicker and contain cells. The most widespread mesogloeal cells are amoebocytes, which also occur in the basal regions of the epithelial layers.

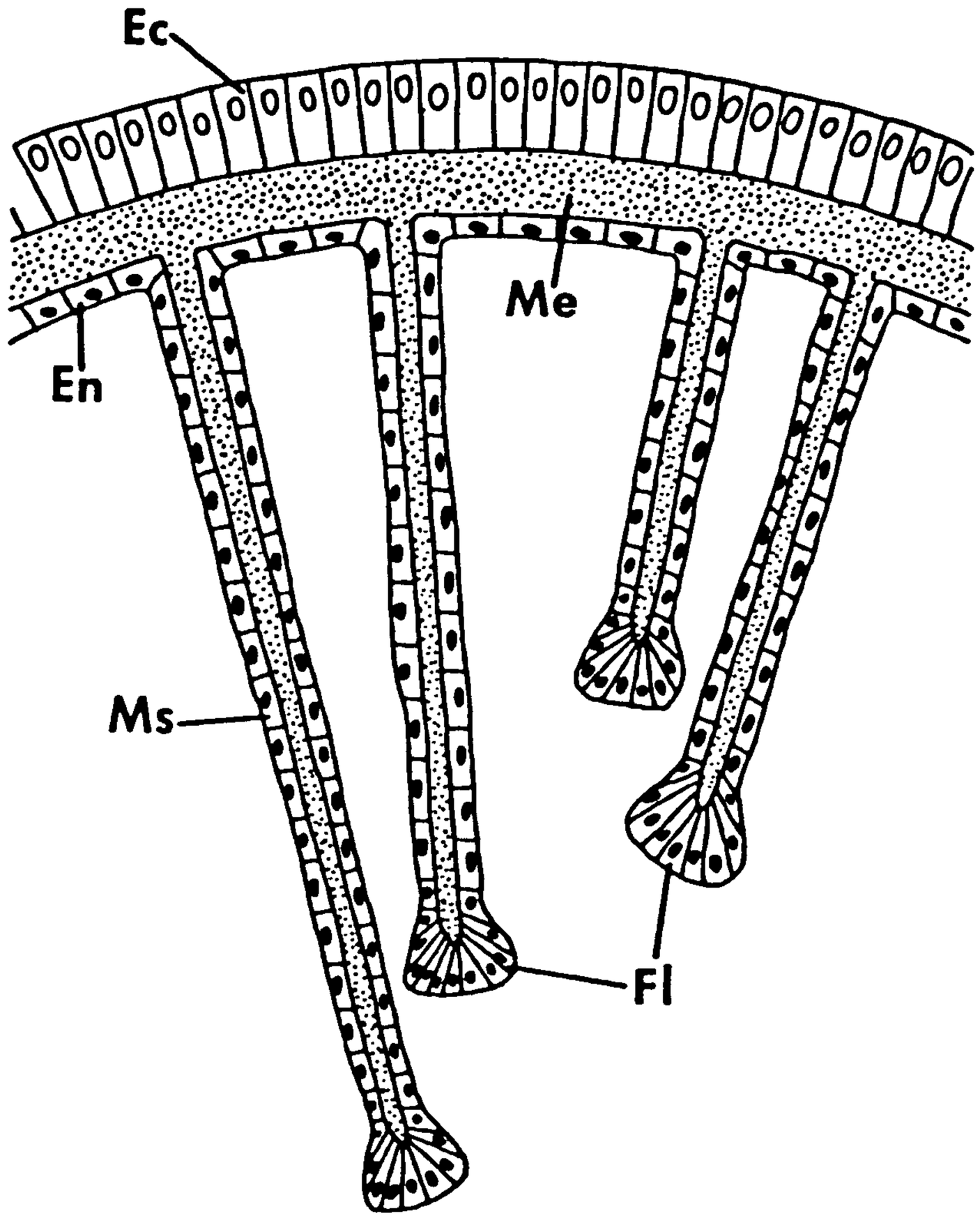
The mesenteries, unlike the body wall generally, consist of two layers of endoderm separated by mesogloea (see Diagram One). Some mesenteries extend from the outer body wall across to the pharynx, and are termed perfect mesenteries, while others do not join with the pharynx and are termed imperfect. The free edge of most mesenteries bears a cord of specialized cells known as the mesenterial or gastric filament. In the upper or oral part of the anemone the filaments have a trilobed appearance in cross section; below about the level of the pharynx they are unilobed. The gonads are situated on the lower portions of the mesenteries. Mesentery and gonad structure will be considered in more detail in Chapter 3, and that of the filaments in Chapter 8.

Diagram 1.

Simplified diagram showing the arrangement of the cell layers in a sea anemone. Part of the anemone is shown in transverse section, at a level through the pharynx (left) and below the pharynx (right). The body wall (top) consists of ectoderm, mesogloea and endoderm. Mesenteries extend centripetally from the body wall, and consist of two layers of endoderm separated by mesogloea. Perfect mesenteries extend all the way to the pharynx, while imperfect mesenteries do not, and bear a thickened filament on their inner edge.



1



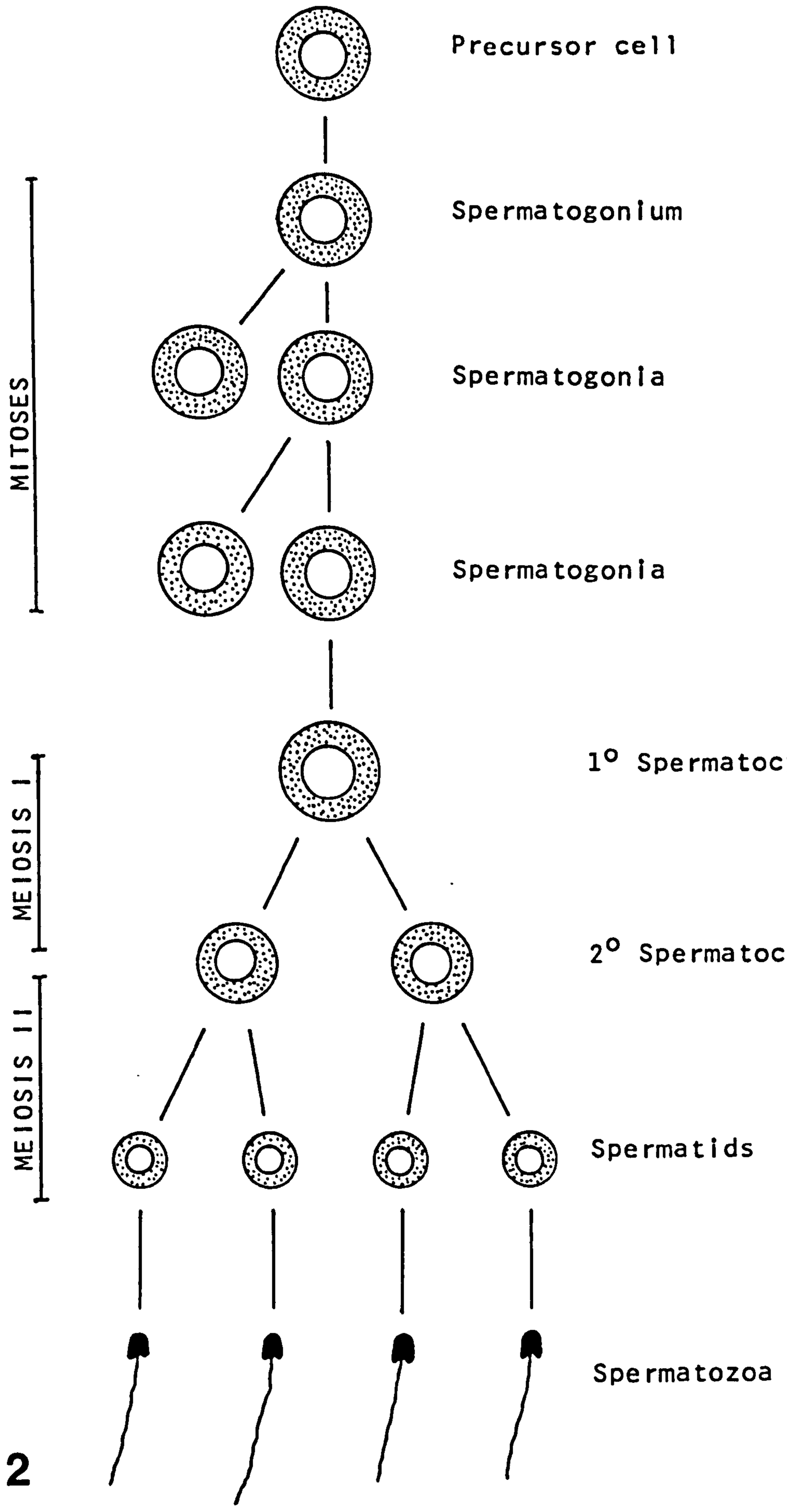
ii) The terminology of gametogenesis

There is now a vast literature dealing with spermatogenesis and oogenesis. No attempt to survey this literature will be made here: more specific areas will be dealt with in individual chapters. Ultrastructural aspects of oogenesis have been reviewed by Norrevang (1968) and Anderson (1974). Spermatogenesis was surveyed by Roosen-Runge (1977), and the structure of spermatozoa by Baccetti and Afzelius (1976). The literature on invertebrate gametogenesis has been recently reviewed in volumes edited by Adiyodi and Adiyodi (1983a, b).

While the end products of gametogenesis vary hugely between different species, there is a measure of consistency in the pattern of stages which are passed through during their formation. The following very brief account is generalized and may be applied to many, but not all, animal groups. Again, it is included primarily to introduce some of the terms which will be encountered frequently in subsequent chapters.

Spermatogenesis is in some respects the more straightforward process and so will be outlined first. The stages involved are shown in Diagram 2. Many animals do not begin active gametogenesis until they reach a particular age or size, but germ cells may be present from a very early age. Many terms have been applied to these precursor cells in different situations, but it is perhaps simplest to refer loosely to them as precursor cells. When active spermatogenesis begins in the male, these cells become mitotically active, and are known as spermatogonia. They undergo several rounds of mitotic division, before some of them become primary spermatocytes, which now divide meiotically. The first meiotic

STAGES OF SPERMATOGENESIS

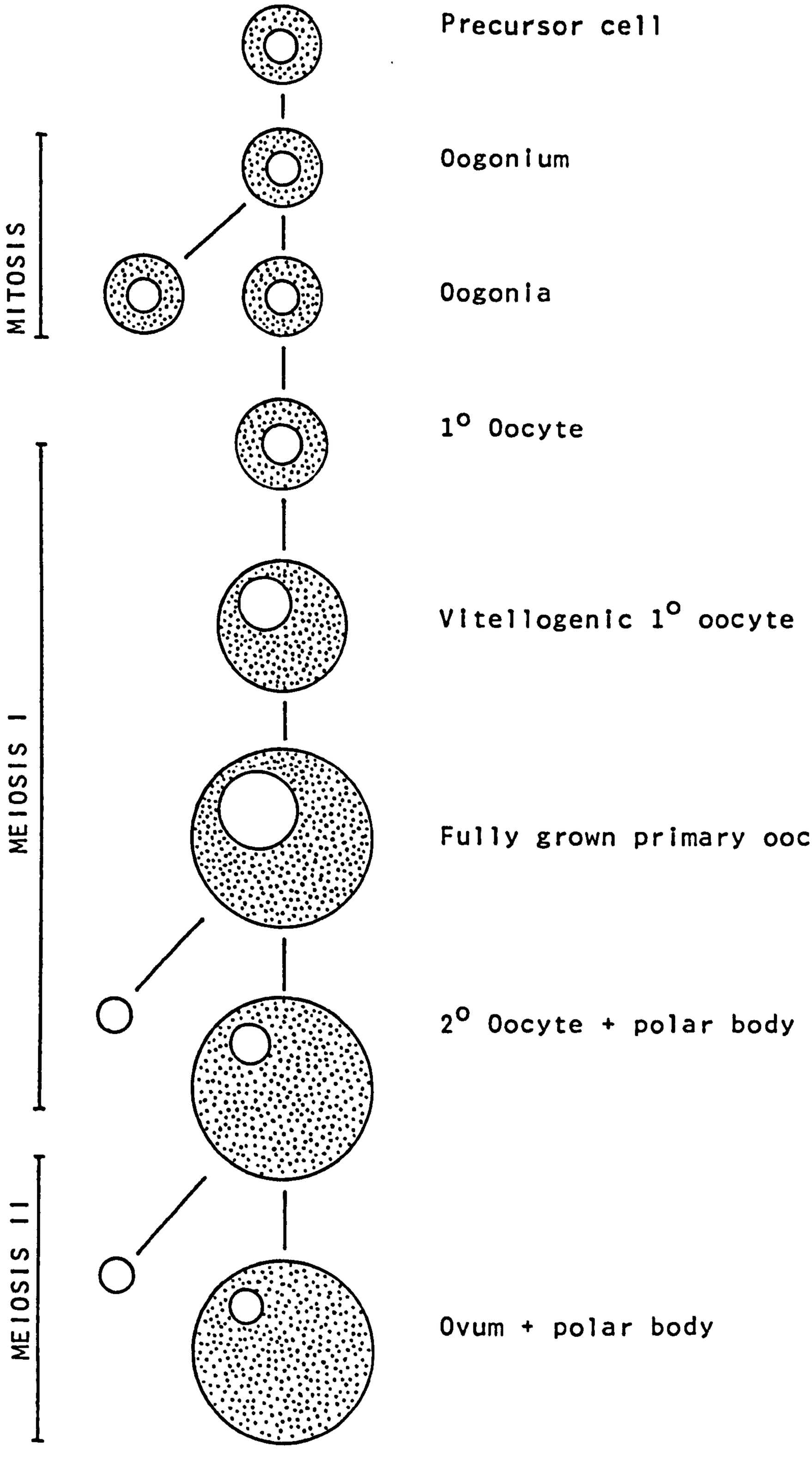


2

division gives rise to a pair of secondary spermatocytes, which then undergo the second meiotic division to produce four cells known as spermatids. The spermatids then differentiate without further division to form the spermatozoa, a process sometimes called spermateliosis. Sometimes the cytoplasmic divisions following mitosis and meiosis may be incomplete, giving rise to daughter cells conjoined by cytoplasmic bridges.

The stages of oogenesis are shown in Diagram 3. Oogonia, again derived from precursor cells, divide mitotically for a period before developing into primary oocytes. Primary oocytes begin meiosis, but the process is usually arrested, often at the diplotene stage of first meiotic prophase. During meiotic arrest, the primary oocyte enlarges and may accumulate reserve materials known as yolk. The process of yolk accumulation is known as vitellogenesis, and may take months or years to complete. When growth is complete, meiosis may be resumed. The distribution of cytoplasm after the first meiotic division is usually very unequal. One daughter cell receives the vast majority of cytoplasm and becomes the secondary oocyte, while the other cell receives very little. It is termed the first polar body, and usually soon degenerates. The second meiotic division follows a similar pattern, producing a large ovum or egg and a second small polar body. The egg or oocyte may be released from the gonad at any stage after its growth is complete; meiosis and polar body formation may thus occur after release. Fusion of sperm and egg may also occur prior to the completion of the meiotic divisions. Fusion of the haploid egg and sperm genomes produces a diploid zygote which then undergoes cleavage to form an embryo.

STAGES OF OOGENESIS



3

During spermatogenesis, most of the extensive reorganization required to fashion the mature spermatozoa takes place after mitotic and meiotic activity has ceased. During oogenesis, however, much of the growth and transformation take place during a pause in the first meiotic division.

Egg and sperm differentiation often take place in close association with other cells not destined to become gametes. These so-called accessory cells may take a variety of forms. Some sperm develop while embedded in the cytoplasm of sustentacular or Sertoli cells, while most oocytes are associated with follicle cells, nurse cells or both. Follicle cells are derived from somatic cells, while nurse cells originate from germ cells, and may show cytoplasmic continuity with the developing oocyte. In many species, these accessory cells make a very significant contribution to gamete development. Interestingly, Campbell (1974a) proposes coelenterates as possible model systems for the study of gametogenesis with minimal or no involvement of accessory cells. The validity of this suggestion will be examined critically in the chapters which follow.

Chapter 2

MATERIALS AND METHODS

1. Obtaining Gonad Material

Actinia fragacea individuals were collected at approximately monthly intervals throughout 1979 and 1980 from a restricted area of rocky shore at Wembury (some 5 miles south-east of Plymouth, Devon). Collection from this site was continued at intervals of 6-8 weeks during 1981. This population appeared to consist of roughly equal numbers of males and females, and samples of 6 animals were taken on each occasion. By late 1981, the population showed signs of depletion, partly as a consequence of sampling and partly due to the covering of some rocks by sand movements during 1981. Further sampling as required for experimental work during 1982 and 1983 was carried out from the shore at Shaldon (approximately 1 mile south-west of Teignmouth, Devon). The Shaldon population contained a preponderance of male animals, and samples of 10 to 15 individuals were taken on each occasion. From both sites, animals were collected haphazardly, and selected simply on the basis of size. Only relatively large individuals were taken, and every anemone collected was found to contain recognizable gonads.

Actinia equina, *Tealia felina* and *Anemonia sulcata* individuals were collected at intervals of 6-8 weeks from the Wembury site during 1981. Again, large individuals were selected. Six *Tealia* and *Anemonia* individuals were taken each time, while the high incidence of non-sexual anemones in *Actinia equina* necessitated a sample size of 20 for this species.

Cereus pedunculatus was sampled at approximately 8-weekly intervals throughout 1980 and 1981. The sampling site was situated near Langstone, some 5 miles east of Portsmouth, and was an area of intertidal mud flat in the upper reaches of

Langstone Harbour. Samples of 8 large individuals were taken on each occasion.

On returning to the laboratory, anemones of all species were placed in closed tanks of aerated seawater at room temperature (usually 17-20^{o}C). All anemones were dissected as soon as possible after collection, usually the next day. Most appeared unharmed by the collection and transportation process. However, *Tealia felina* individuals were often difficult to remove from the substratum, and many suffered damage, usually to the pedal disc, during collection. Some of these anemones showed signs of morbidity on dissection, often including a darkening of the mesenteric filaments. Throughout the study, difficulty was experienced obtaining apparently healthy, well-fixed gonad material for this species.

Actinia fragacea, *A. equina* and *Anemonia sulcata* individuals were dissected by making two cuts at right angles across the pedal disc, and pinning the anemone out, oral disc downwards, in a wax-bottomed dish. As the pedal disc is everted and spread out, the mesenteries are clearly visible radiating from the pharynx. The gonads may be removed from the mesenteries using fine scissors. It was found to be important to perform the dissection rapidly and smoothly, since severe or prolonged disturbance of the mesenteries usually results in the production of large amounts of opaque sticky mucus, which makes further dissection difficult or impossible.

Cereus and *Tealia* individuals could not be pinned out in this way, and dissection of these anemones was less methodical. They were usually simply cut in half longitudinally and gonad tissue was removed as and when it became visible

during probing of the inside of the anemone.

2. Preparation for Microscopy

Gonad material obtained as above was prepared for light microscopy, transmission electron microscopy and scanning electron microscopy by the following procedures. Details of solutions and reagents used in this section are given in Appendix 1.

a) Fixation

All gonad pieces were fixed immediately after removal by immersion in fixative consisting of 3% glutaraldehyde and 3% sodium chloride in a 0.1 molar sodium phosphate buffer, pH 7.4. Fixation was for 2h at room temperature, after which the tissue was stored in buffer at 4°C.

Fixation in PIPES-buffered glutaraldehyde, as recommended by Baur and Stacey (1977), was also tried. Tissue preservation at LM and EM levels was generally excellent, but with EM the cytoplasmic matrix appeared much more dense than with phosphate buffered fixative. While this may have been due to improved retention of cytoplasmic components, it made the visualization of organelles more difficult. For this reason, PIPES buffer was not adopted for routine use.

b) Preparation for light microscopy

Gonad material intended for light microscopic examination was fixed and stored as described above, then trimmed as required with a razor blade and dehydrated as follows:

70%	ethanol/water	30min
90%	" "	30min
95%	" "	30min

The material was infiltrated and embedded in 'JB4' plastic

as follows (details in Appendix 1).

Soaked in catalysed solution A for 2 h,

Rinsed in complete resin mix for 5 min,

Embedded in complete resin in LKB 'Easymoulds'.

'Easymoulds' can be stacked, to exclude air during polymerization of the resin. Polymerization was usually complete after 3 h at room temperature.

Sections were cut at 2 μm thickness using glass knives on an LKB Pyramitome. The sections were transferred to water droplets on glass slides and dried on a hotplate. The sections were routinely stained briefly with toluidine blue, while a small number were stained using haematoxylin and phloxine. The stained sections were photographed using Wild M20 or Leitz Dialux photomicroscopes and Ilford Pan F 35mm format film.

c) Preparation for transmission electron microscopy

Material for routine TEM was fixed and stored as described above, trimmed as required, cut into small pieces and processed as outlined below.

1% osmium tetroxide in phosphate buffer	1 h
Buffer rinse	30 min
70% ethanol/water	10 min
90% " "	15 min
95% " "	15 min
100% ethanol	15 min
100% "	15 min
Epoxypropane	15 min
Epoxypropane	15 min

The pieces were infiltrated in 'Emix' epoxy resin, medium

hardness mix, as follows:

1:1 epoxypropane/epoxy resin	30 min
Epoxy resin	1.5 h

The pieces were then embedded in epoxy resin in 'Micromould' capsules and polymerized at 60°C for approximately 16 h.

Some of the earlier material was subjected to tertiary fixation (or *en bloc* staining) with uranyl acetate. For these cases the procedure was as follows:

1% osmium tetroxide in phosphate buffer	1 h
Distilled water	30 min
1.5% aqueous uranyl acetate	1 h

followed by dehydration and embedding in the usual way. Material prepared in this way showed excellent preservation of much fine structure, but exhibited gross changes in the appearance of glycogen. These changes were similar to those described for a range of tissues by Vye and Fischman (1970). With experience, the altered glycogen could still be identified in sections with little difficulty, but since glycogen proved to be a major constituent of many cells involved in gametogenesis, the use of uranyl acetate as a tertiary fixative was discontinued.

After embedding, sections were cut using glass or diamond knives on an LKB Ultratome III ultramicrotome. The sections were flattened using chloroform vapour and collected on uncoated copper grids with mesh sizes between 200 and 400 per inch, depending on the structures under investigation.

The sections were double stained with uranyl acetate and lead citrate (detailed in Appendix 1). The grids bearing sections were immersed in the staining solutions in solid

watch glasses for 15 min in each case, and rinsed after each solution by repeated dipping into distilled water.

Stained sections were examined using a Philips EM300 electron microscope, and photographed using Kodak 4489 or 4463 electron microscope film. Negatives were developed using Kodak D19 developer, and fixed using Ilford Hypam rapid fixer.

d) Preparation for scanning electron microscopy

Material intended for SEM examination was fixed, osmicated and dehydrated as for TEM. After dehydration to 100% ethanol, it was treated as follows:

1:1 100% ethanol/acetone mixture	30 min
100% acetone	30 min
100% acetone	30 min

From acetone, the material was dried by the critical point method using Polaron apparatus. Pieces were attached to SEM specimen stubs using double sided adhesive tape or 'silver Dag' conductive paint. The stubs were then coated with a gold/palladium mixture using a Polaron sputter coater. They were examined using a JEOL JEM 35 scanning electron microscope, and photographed using Ilford FP4 film which was developed in ID 11 developer and fixed in Hypam.

All negatives were printed using the Ilford Ilfoprint automatic processing system.

e) Handling of eggs, embryos and larvae

Initially, eggs, embryos and larvae were processed individually for LM, TEM and SEM. However, their small size made the manipulations required slow and tedious, and some loss of material often occurrred during processing. To facilitate the rapid processing of large numbers for LM and TEM, an agar embedding method was devised, and a small chamber was made to contain specimens for SEM during critical point drying.

Agar embedding

Several procedures were attempted, but the following method proved simple and effective. The specimens were fixed and osmicated as normal for TEM. 100-300 embryos were then transferred in a small volume of buffer to an 8 mm BEEM-type embedding capsule from which the cap had been removed. The specimens were allowed to settle to the pointed tip of the capsule, and as much of the buffer as possible was carefully drawn off with a Pasteur pipette. The capsule was then allowed to slide gently to the bottom of a round-bottomed 15 ml glass centrifuge tube. The tube and capsule were then placed in a rack in a water bath at 60^{o}C for a few minutes. Three to four drops of molten agar (1.5% agarose in distilled water) were added to the capsule using a warmed pipette, and the tube was gently agitated to mix the agar with the buffer and the specimens. The tube was then replaced in the water bath, and allowed to stand until the specimens again settled to the tip of the capsule. The tube was then removed from the water bath, wiped dry and placed in a refrigerator for some 15 min, during which the agar solidified. The capsule was then removed from the tube, cut open with a razor blade,

and the agar block containing the specimens removed. The osmicated eggs or embryos could be clearly seen within the agar, which could now be trimmed and cut into smaller pieces. These pieces, each containing 20-50 closely-packed specimens, could then be dehydrated and embedded for LM and TEM in the same way as pieces of gonad tissue.

Handling for SEM

Eggs, embryos and larvae for SEM were fixed, dehydrated and taken to acetone as described for gonad material above. For critical point drying, however, they were placed in a small, porous, plastic chamber rather than the larger metal gauze chamber supplied with the equipment. The small chamber consisted of a polythene tube, made by cutting the tapered end off an 8 mm BEEM-type embedding capsule, sealed at each end by a small piece of 64 μm pore-size nylon mesh. The mesh was held in place at each end by a BEEM-capsule lid, the central part of which had been cut away using a cork borer.

The specimens were pipetted into the open chamber under acetone, and the gauze and lid replaced. The whole chamber was then transferred to the critical point drying apparatus. After drying, the cap and gauze were removed from one end of the chamber, and the specimens were sprinkled out onto a stub previously coated with conductive paint or adhesive tape. They were then coated and examined in the usual way.

3. Removal of Gonad Tissue by Biopsy

For most experimental procedures it was useful to know the sex of the anemones to be used before the start of the experiment. For this purpose, the biopsy procedure developed by Carter and Funnell (1980) was employed. The selected anemone is removed from its aquarium and placed upright in a

small dissecting dish. This disturbance usually causes the anemone to contract, otherwise it may be encouraged to do so by prodding, since extended tentacles make the operation more difficult. A small vertical incision is then made in the body wall of the lower part of the column; with practice, the incision need only be 3-5 mm long. In some cases, mesentery and gonad tissue may protrude through the incision spontaneously but more often they must be pulled out using fine forceps. A small piece of gonad may then be excised and fixed; usually the gonad must be sectioned and examined microscopically before the sex of the anemone can be determined. Carter and Funnell originally closed the incision by means of a suture, but it seems that small incisions heal more rapidly if left unsutured. Provided the operation has been carried out carefully, the anemone usually opens and recovers within a few minutes. If left, the wound heals within about 10 days.

4. Maintenance of Gonad Tissue *in vitro*.

For some experiments, it was convenient to work with isolated gonad tissue maintained *in vitro* rather than whole anemones. Gonad tissue was removed by dissection or biopsy, and placed in filter sterilized sea water (SSW) to which antibiotic (crystamycin - see Appendix II) had been added. The gonad pieces were then carefully trimmed free of filament and mesentery tissue using a razor blade. Using sterilized forceps and aseptic technique, the gonad pieces were washed by swirling in five separate dishes containing SSW and antibiotic. They were finally transferred to sterile glass vials of 10 ml capacity containing approximately 3 ml of SSW and antibiotic, and sealed with airtight polythene caps. The vials were kept at room temperature, but away from direct

artificial light or sunlight.

By this method, isolated gonads could be maintained for 14 days and appear perfectly normal when sectioned for light microscopy. Some gonads appeared externally healthy after 4 weeks, but these have not been examined histologically.

Very small gonad pieces do not survive well by this method, and nor do those contaminated with other tissues. Those with sizeable pieces of mesentery attached tend to become covered in mucus and deteriorate. Contaminating mesenteric filament tends to disintegrate and render the cultures liable to infection, principally by fungi. With care, however, the failure rate for gonad material was minimal.

5. Nutrition Experiments

a) Uptake of fish blood cells

Several red gurnard, between 1 and 3 kg in weight, were obtained fresh from a local fish market. These are one of the few species of fish landed intact rather than eviscerated at sea. Blood clots were removed by dissection. Clots of reasonable size (3-5 mm^3) were found within and around the hearts, and less reliably elsewhere. The clots were stored at 4^oC overnight.

Nine *A. fragacea* individuals were collected from Wembury in late October 1981, and maintained in individual glass jars with a daily change of sea water for 6 days. On the morning of the experiments, the water was changed, which usually induces the anemones to open fully. Thirty minutes later, each anemone was fed one or more fish blood clots. The clots varied in size, but an attempt was made to feed

each anemone a roughly similar quantity. All the anemones ingested the clots readily.

Three anemones were dissected at each of 3 times: at 2, 6 and 24 h post feeding. Samples of column body wall, tentacle, mesentery, lower and upper filament and gonad were taken from each animal, fixed and processed for light microscopy. Sections were stained with toluidine blue. Some fixed material was stored and, after preliminary LM observations had been made, some gonad and upper filament samples were processed for TEM.

b) Uptake of horseradish peroxidase

The ability of gonad epithelial cells to take up horseradish peroxidase (HRP) from the external medium was investigated using isolated gonad pieces maintained *in vitro*. Details of the reagents used are given in Appendix III.

Several gonads were removed by dissection from a known female anemone. The gonads were cut into smaller pieces (some 2 mm in length) and washed in sterile sea water (SSW) with antibiotic as described earlier. The pieces were then incubated at room temperature in a solution of horseradish peroxidase (10 mg/ml) in SSW without antibiotic. Four to six gonad pieces were incubated in each of several glass vials containing 2.5 ml HRP solution for either 30 min or 2 h. Control pieces were incubated in SSW only.

After incubation, the gonad pieces were fixed in glutaraldehyde as usual, and stored overnight in buffer at $4^{o}C$. They were then trimmed and cut into smaller pieces, less than 0.5 mm cubes, and peroxidase activity was demonstrated by the following method. Hanker-Yates reagent was used instead of the more usual diaminobenzidine because it is thought to be more specific and is non-carcinogenic

(Hanker *et al*, 1977). All incubations were carried out at room temperature.

i) Gonad pieces were pre-incubated in Hanker-Yates reagent in tris/HCl buffer for 45 min.

ii) Pieces were incubated in buffered Hanker-Yates reagent with hydrogen peroxide for 15 min. Some exposed pieces were incubated in medium lacking peroxide to act as controls.

iii) All pieces were washed in 2 changes of phosphate buffer, 10 min in each.

Material for LM examination was dehydrated and embedded as usual. Material for EM was osmicated then processed in the usual way, but stained for 5 min in lead citrate only.

The experiment was repeated using a similar procedure, but, to ensure complete penetration of the reagents, the tissue pieces were cut into 50 μm slices prior to incubation in Hanker-Yates reagent. The modified procedure was as follows:

i) Gonad pieces were obtained, exposed to HRP and fixed in glutaraldehyde as before.

ii) Washed overnight in 0.1 M phosphate buffer containing 30% sucrose at $4^{o}C$.

iii) The pieces were frozen and cut into 50 μm thick slices using a sledge microtome fitted with a 'Pelcool' freezing attachment.

iv) Rinsed in 0.1 M phosphate buffer containing 3% NaCl for 10 min.

v) Pre-incubated in buffered Hanker-Yates reagent for 20 min.

vi) Incubated in buffered Hanker-Yates reagent with hydrogen peroxide for 15 min.

vii) Rinsed in buffer and processed for LM and EM as before.

c) Demonstration of acid phosphatase activity

Acid phosphatase enzyme activity in male and female gonads was investigated using the following Gomori-based method, adapted from Lewis and Knight (1977). Details of all the reagents used are given in Appendix IV. All procedures were carried out at room temperature unless otherwise indicated.

i) Gonad pieces were removed by dissection from known male and female anemones.

ii) Fixed in cacodylate-buffered glutaraldehyde for 2 h at 4°C.

iii) Rinsed in cacodylate buffer overnight at 4°C.

iv) Frozen and cut into 50 µm slices using a sledge microtome fitted with a 'Pelcool' freezing attachment.

v) Slices rinsed in 0.2 M tris/maleate buffer for 10 min.

vi) Incubated for 1 h in a medium consisting of:

0.2 M tris/maleate buffer	10 ml
0.1 M sodium β-glycerophosphate	2 ml
0.02 M lead nitrate	3 ml
Distilled water	10 ml

Control slices were incubated in a) medium lacking sodium β-glycerophosphate substrate, or b) complete medium containing 0.01 M sodium fluoride as an inhibitor of acid phosphatase activity.

vii) Rinsed in cacodylate buffer for 30 min.

viii) Processed for TEM in the usual way but stained for 5 min in lead citrate only.

d) Uptake of dissolved precursor molecules

The ability of gonad tissue to take up and utilize dissolved precursors was investigated using radiolabelling techniques. Isolated male and female gonads were incubated with tritiated glucose or leucine, and sites of precursor incorporations were demonstrated autoradiographically. The following procedure was employed:

i) Gonads were removed by dissection from animals of known sex, and rinsed in SSW with antibiotic as described earlier.

ii) Incubated at room temperature in radiochemical dissolved in SSW with antibiotic. Loading times ranged from 5 min to 3 h.

iii) Rinsed in SSW and placed in low concentration of unlabeled ('cold') chemical in SSW with antibiotic. 'Cold chase' times ranged from zero to 72 h.

iv) Fixed in phosphate buffered glutaraldehyde for 2 h.

v) Rinsed in buffer for 3 days.

vi) Dehydrated and embedded for LM in the usual way.

vii) Sections cut at 2 μm thickness, mounted on acid washed glass slides and allowed to dry down firmly.

viii) Slides coated by dipping in Ilford K2 nuclear research emulsion. Details of the dipping procedure are given below.

ix) Slides sealed in light tight box and kept at $4^{o}C$ for exposure period of between 4 and 7 days.

x) Slides developed, fixed, washed and dried. Details of the development procedure are given below.

xi) Sections stained briefly with toluidine blue, dried and mounted in Euparal.

Details of the radiochemicals employed are given in Appendix V. They were normally used at a final radioactive concentration of 10 uCi/ml. Some unincubated control gonads were fixed and processed exactly as above to test for positive chemography. A small number of slides from these control gonads were exposed to day-light after coating but before the exposure period to test for negative chemography.

Coating procedure

The slides were marked with a wax pencil to indicate which side had the sections, and the depth to which the slide had to be dipped to ensure the sections were coated.

In a darkroom, using an Ilford S902 safelight, the emulsion was prepared; 6 ml of K2 emulsion were melted in a measuring cylinder in a water bath at 43^{o}C, then mixed with 6 ml of distilled water and 0.125 ml of glycerol. The diluted emulsion was stirred gently, poured into the dipping vessel and allowed to stand in the water bath for 2 min to allow air bubbles to escape. The slides were dipped into the emulsion and withdrawn slowly using the simple machine shown in Diagram 4.

After dipping, the reverse sides of the slides were wiped with tissue, and they were placed on a cooled glass plate in a small, light-proof metal cabinet and left for 1 h. The slides were then placed in small plastic slide boxes, which were left open in the cabinet with a tray of silica-gel desiccant overnight.

Next day a small muslin bag of desiccant was placed in each of the slide boxes, which were closed up, sealed with tape, placed inside a larger cardboard box and put in a refrigerator for the required exposure period.

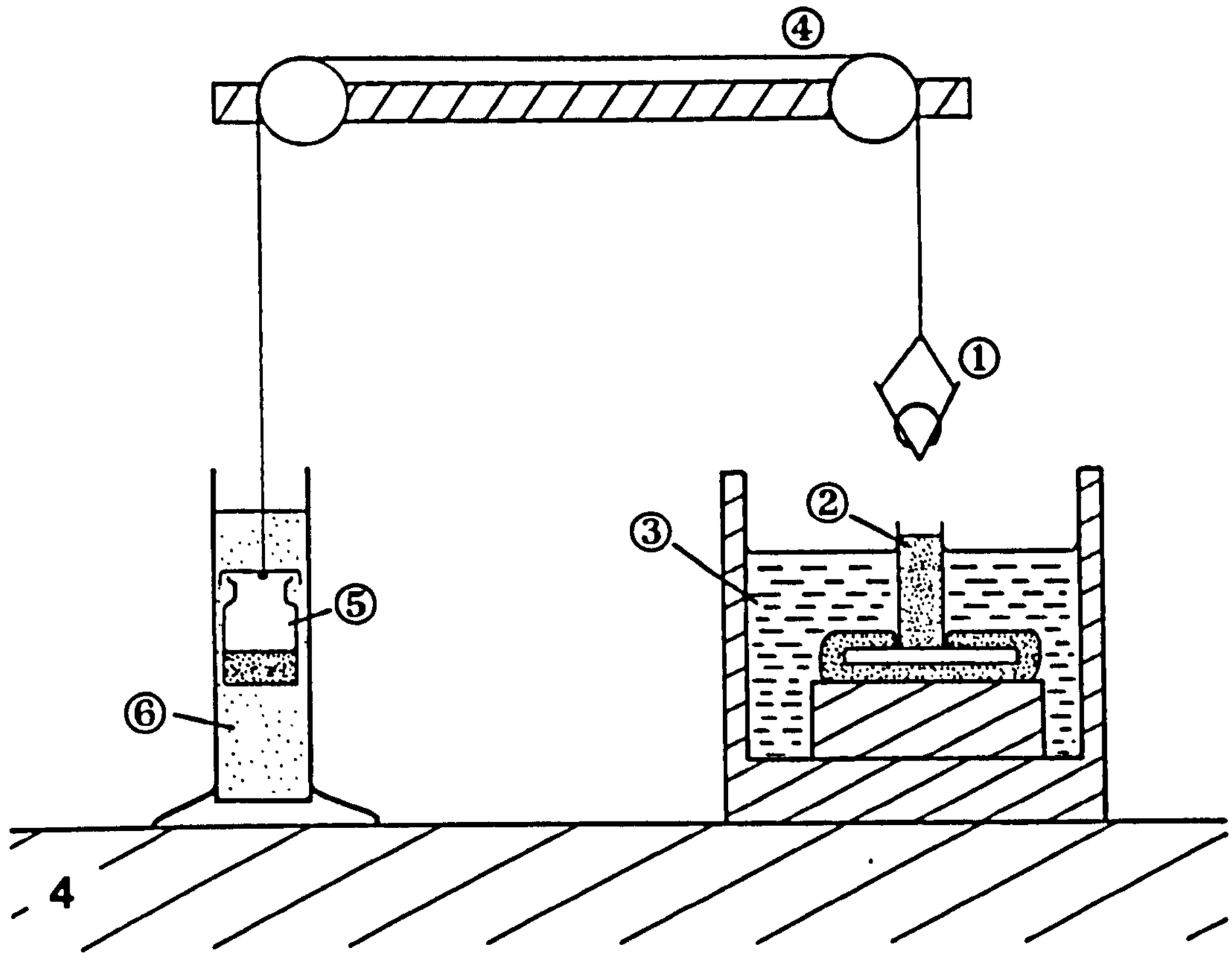


Diagram 4.

The simple dipping machine used to coat sections with emulsion for autoradiography. The slide to be coated is fastened in a bulldog clip (1) and immersed in the prepared emulsion (2) in a small plastic chamber, which is itself maintained in a water bath (3) at 43°C. A length of nylon monofilament (4) is attached to the bulldog clip, passes over 2 pulley wheels and is connected to a small glass vial containing mercury (5). This vial is allowed to fall through a column of glycerol in a measuring cylinder (6), thereby withdrawing the slide from the emulsion. The rate of withdrawal of the slide can be adjusted by altering the amount of mercury in the glass vial.

Development procedure

After the required exposure period, the slides were removed from the boxes and processed as follows:

Developed for 8 min in Kodak D-19, diluted 1+1,
Rinsed for 1 min in distilled water,
Fixed for 1 min in Ilford Hypam, diluted 1+4,
Washed for 15 min in running tap water,
Rinsed for 1 min in distilled water,
Dried on a hotplate at 30°C for 1 h or more.

Chapter 3

GONAD STRUCTURE AND GAMETOGENIC CYCLE

INTRODUCTION

It has been suggested that sea anemones do not possess discrete gonads, merely diffuse areas of tissue in which germ cells may be found (Rostron, 1970; Dunn, 1975). Nevertheless, the term gonad is widely employed, and some justification for its use will be given later in this chapter. The structure of sea anemone gonads, as opposed to gametes, has received little recent attention, and the only information available at the ultrastructural level is a brief mention by Clark and Dewel (1974). An understanding of the environment in which the germ cells develop may be very important, however, so the structure of the gonad epithelial cells in *A. fragacea* will be considered in this chapter. Small areas of gonad epithelium associated with each oocyte or testicular cyst appear to differentiate into specialized structures known as trophonemata (Hertwig and Hertwig, 1879; Nyholm, 1943). The structure of these trophonemata will be considered in some detail in Chapters 8 and 11, and will not be dealt with here.

The gonads of many marine invertebrates undergo cyclical changes in their development, and there is an obvious advantage in synchronizing the maturation of eggs and sperm. Among sea anemones, an annual cycle has been reported for *Anthopleura elegantissima* (Jennison, 1979), a monthly one for *Actinia equina* (Rostron, 1970; Chia and Rostron, 1970) and no obvious cycle at all for the largely subtidal *Epiactis prolifera* (Dunn, 1975). In *A. fragacea*, the cycle is perhaps more easily followed with the light microscope rather that at the EM level, where increased detail may obscure underlying trends. The following account is based on light microscope observations

of regular samples over a two year period. The pattern was very consistent between the two years, and later occasional sampling also confirmed the picture. Many of the processes mentioned here will be considered in greater detail in later chapters, to which this will serve as a brief introduction.

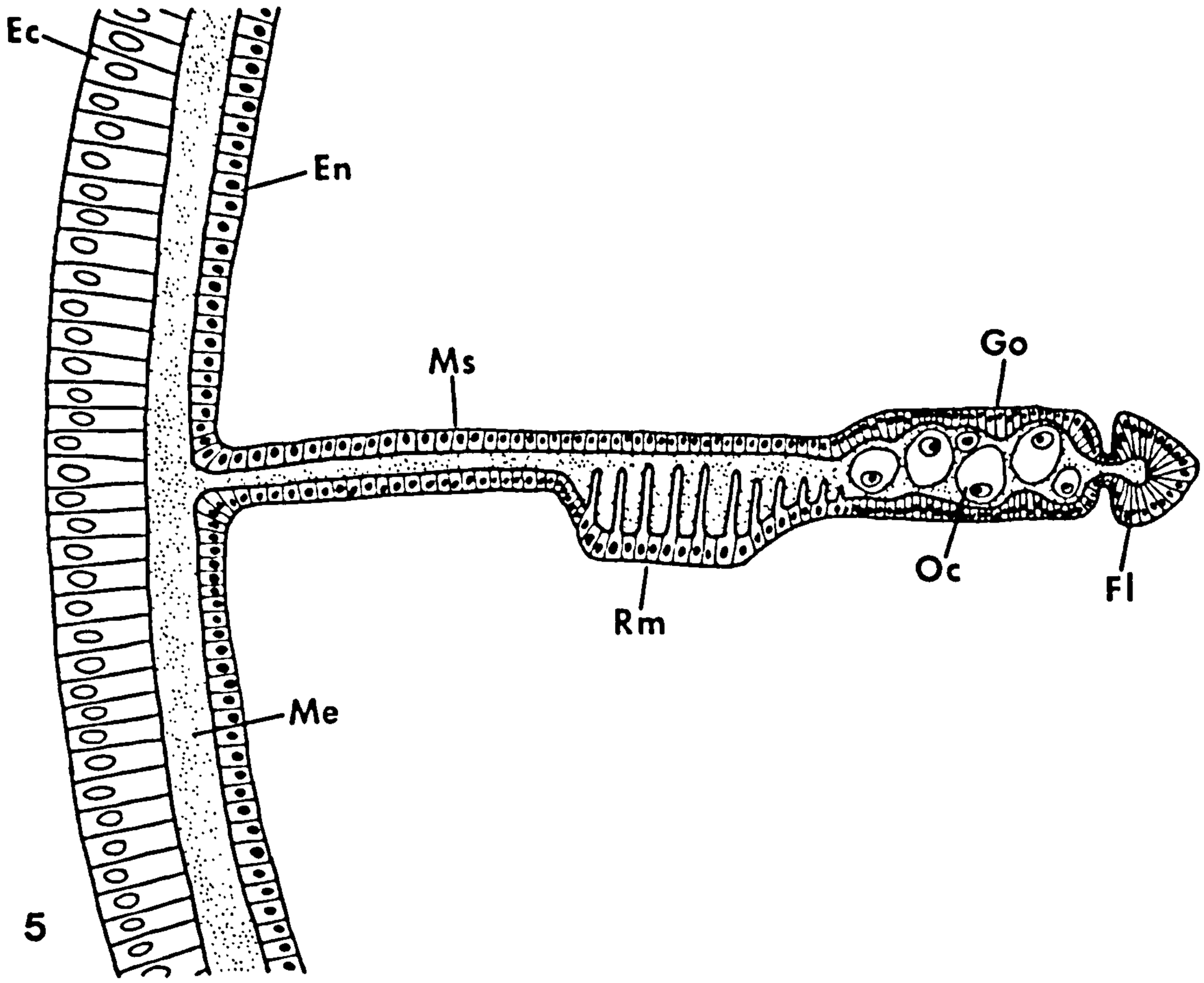
RESULTS

1. Gonad Structure

In *A. fragacea*, as in other sea anemones, the gonads are located on the mesenteries. They lie between the mesenteric retractor muscles and the gastric filaments (see Diagram 5). Not all mesenteries bear gonads, and they only occur on the lower portions. Each gonad is thus an elongate, ribbon-like structure, which, like the adjoining filament, tends to be longer than the rest of the mesentery and so is convoluted. After fixation, gonads tend to contract into a regular series of folds (Fig. 3.1).

Like the remainder of the mesentery, the gonad consists of two layers of endodermal epithelial cells separated by a layer of mesogloea (Fig. 3.2). The germ cells arise in the epithelial layers, but the later stages of their development take place in the mesogloea. The gonad epithelia are composed largely of rather unspecialized columnar epithelial cells. Mucus secreting cells are also present (Fig. 3.3), but in much smaller numbers than in the rest of the mesentery. Gland cells and nematocysts are also occasionally found. The appearance of the gonad epithelia appears the same in male and female gonads, and also remains constant throughout the gametogenic cycle.

The apical surfaces of the gonad epithelial cells bear cilia and microvilli. The density of the microvillar covering varies from region to region, but they are often closely packed (Fig. 3.4). The base of each cilium is surrounded by a ring of 9-12 microvilli, some which appear to be flattened into sheets rather than circular in cross section (Fig. 3.5). The space between the base of the cilium and the microvillar



5

Diagram 5.

A simplified diagram of a single, gonad-bearing mesentery, sectioned transversely. The mesentery is attached to the body wall (left), and bears a thickened region containing well-developed muscle tails, known as the mesenteric retractor muscle. The gonads are located between the retractor muscle and the inner edge of the mesentery which bears the filament (right).

the epithelium with the mesogloea, the basal processes may be expanded and contain muscle microfilaments. The degree to which these muscle processes are developed varies greatly. In some regions they are virtually non-existent, while in others they are well developed and joined by desmosomes to form continuous sheets (Fig. 3.8). Where the gonad borders the mesenteric retractor muscle, the processes may be large and folded to form long muscle tails which extend into the mesogloea, which may be relatively thick (Figs. 3.9, 3.10). The slender processes connecting the muscle processes to the epithelial cell bodies can just be distinguished running through the central regions of the tails.

The epithelial cell bases rest against a thin, finely fibrillar basal lamina which separates them from the mesogloea proper (Figs. 3.8-3.10). The appearance of the mesogloea also shows considerable variation. Usually it is perhaps 10-15 μm in thickness, and contains numerous, randomly orientated collagen fibres. Around developing germ cells, it may be much thinner, perhaps 2-5 μm, and contain relatively few fibres. In regions where the muscle processes are well developed, it may be 100 μm or more across, and contain closely packed fibres arranged in parallel bundles. Amoebocytes and their processes are often seen in the mesogloea (Fig. 3.9), but are also common in the basal portions of the epithelia. Amoebocytes are described and illustrated in Chapter 6 (Figs, 6.203-6.210). No structures which could be identified with any degree of confidence as nervous elements were found in male or female gonad tissue.

2. Gametogenic Cycles

A cycle is more obvious in male than female gonads, so the male will be considered first.

Male germ cells, thought to be spermatogonia, first appear in the gonad mesogloea during September or October, and are found as isolated small groups (Fig. 3.11) scattered throughout the gonad. During this period, groups of prospermatogonia can occasionally be seen among the epithelial cell bases, and rarely can be observed entering the mesogloea (Fig. 3.12). By December, the groups of spermatogonia have increased in size and number, forming testicular cysts which occupy most of the gonad mesogloea (Fig. 3.13). By February, the cysts may be 80 μm in diameter, and the cells near their centres begin visible differentiation into spermatids and spermatozoa (Fig. 3.14). During the spring, the cysts enlarge and the more advanced developmental stages occupy an increasing proportion of their volume. By May, spermatogonia are restricted to a thin rim around the periphery of each cyst. The spermatids and spermatozoa become arranged in strings or cords, with their tails all directed towards one side of the cyst (Fig. 3.15). Release of sperm occurs during June and July, and during this period, partially emptied testicular cysts may be found (Fig. 3.16). No instances where the gonad epithelia appeared disrupted were observed. It seems that sperm release occurs through specific structures in the epithelium known as the trophonemata rather than by general breakdown of the gonad structure. Also at this time, cysts which appear vacuolated and which have lost the orderly arrangement of sperm heads and tails occur. Sperm heads may be found among the epithelial cells, and it is thought that

these cysts may be breaking down and the gametes undergoing resorption (Fig. 3.17). By August, all the gametes have been shed or resorbed, and the gonads appear to be devoid of germ cells (Fig. 3.18). Localized dilations of the mesogloea may indicate sites previously occupied by testicular cysts. The gonads remain empty until the new season's batch of germ cells arises and enters the mesogloea during September or October.

The female gametogenic cycle is rather less obvious. There is no clear-cut starting point, but it is perhaps simplest to begin in the autumn, as was done for the male. By September, the gonad mesogloea contains many small oocytes, ranging between 20 and 60 μm in diameter (Fig. 3.19). Most are in the early stages of vitellogenesis, and both their nuclei and cytoplasm appear dense. Their yolk materials are less strongly basophilic, so as vitellogenesis proceeds, less dense areas appear in the cytoplasm. By December, the gonads contain some oocytes up to 80 μm in diameter, and containing considerable amounts of yolk, while others may be only 30 μm and contain very little (Figs. 3.20, 3.21). The light microscope appearance of an oocyte during active vitellogenesis is shown in Fig. 3.22. A band of dense, basophilic material partially surrounds the nucleus, and areas of similar material occur in the cytoplasm. The relatively unstained areas close to the nucleus are thought to represent large glycogen deposits. The cytoplasm appears heterogeneous and contains areas of moderately stained yolk material. Oocyte growth proceeds during winter and spring, and by April, some oocytes have reached their full size of some 150 μm (Fig. 3.23). The cytoplasm of fully grown oocytes appears more homogeneous and less dense than that of vitellogenic ones, being packed with

yolk and containing few basophilic regions (Fig. 3.24). During May, and possibly June, the largest oocytes apparently remain quiescent while smaller ones continue growth, so a degree of 'catching up' occurs. Spawning takes place during June and July, again with no apparent damage or disruption of the gonad epithelia. In the population studied, not all the oocytes were ever spawned, and a number remained, fragmented within the gonad, and were resorbed during July and August.

Throughout the period from May to October, small oocytes arise in the endoderm and migrate into the mesogloea (Fig. 3.25) Thus, they may enter mesogloea filled with large oocytes, relatively empty following spawning, or filled with large oocytes undergoing resorption (Fig. 3.26), depending on the time of entry. This extended period of recruitment into the mesogloeal oocyte population contributes to the asynchrony in development seen even within a single gonad. The asynchrony is reduced prior to spawning by the 'catching up' by small oocytes, but by then the picture is complicated by the appearance of the next season's batch of small oocytes.

It thus appears that both sexes show an annual cycle of development. Sperm production appears to take 8-10 months, while oocyte growth may take rather longer, perhaps 10-14 months. No hermaphrodite gonads or individuals were encountered, and the sex ratio of anemones taken from the Wembury site was approximately 1:1. However, the samples taken from the Shaldon site showed a preponderance of male individuals. Of the 43 animals taken from this site and examined hitologically, 28 were male and 15 female. However, these figures should not be taken as a reliable guide to the sex ratio of the population generally (see Discussion).

DISCUSSION

The location and structure of the gonads in *A. fragacea* appear broadly consistent with the classical descriptions given by Stephenson (1928) and Hyman (1940). However, the cellular composition of the gonad epithelia is markedly different from the neighbouring filament or mesentery tissue. In the male, the gonads persist as morphologically distinct structures, even when they apparently contain no germ cells. Thus the view that sea anemones do not possess discrete gonads (Dunn, 1975) or that gametes develop in the general mesentery mesogloea (Rostron, 1970) needs to be treated with some caution.

The gonad epithelia are principally composed of rather unspecialized cells, known as musculo-epithelial cells or supporting cells (Stephenson, 1928). Each of these cells bears a single cilium, surrounded basally by a ring of microvilli. A similar arrangement was found in gonads of the anemones *Bunodosoma cavernata* and *Metridium* sp. by Clark and Dewel (1974). Similar or related structures have been reported from anthozoan planula and adult ectoderm (Lyons, 1973; Chia and Crawford, 1977; Chia and Koss, 1979). Lyons (1973) pointed out the parallels between these structures and those of sponge collar cells or choanocytes. Variations on the basic pattern of a cilium surrounded by a ring of cylindrical processes have been found in several coelenterate epithelia, and are usually thought to have a sensory function (Tardent and Schmid, 1972; Peteya, 1975; Cormier and Hessinger, 1980; Kinnamon and Westfall, 1984). In *A. fragacea*, as in many other species (Chapman, 1974), the cilium arises from a basal-body-rootlet complex. These structures have been recently examined in some detail from sea anemone pharynx and siphonoglyph epithelia by

Holley (1982, 1983, 1984). He proposes that they function to maintain a common basal orientation between cilia in spite of deformation of the underlying epithelium. The epithelia he studied were highly specialized for the propulsion of water or mucus, with narrow cell apices and hence a high ciliary density. Whether the cilia of gonad epithelia, where the cell apices are much wider, behave in a similar way remains to be established. However, the structure of the basal apparatus appears identical from the various epithelia.

The finding of numerous coated pits, coated vesicles and phagosomes in the apical cytoplasm of the gonad epithelial cells suggests that they are involved in the uptake of nutrient material from the fluid in the gastrovascular cavity. This possibility is explored further in Chapter 8.

In *A. fragacea*, the development of oocytes appears to be only loosely synchronized, even within a single gonad, for much of the gametogenic cycle. Similar asynchronicity has also been reported for the anemones *Peachia quinquecapitata* (Spaulding, 1974), *Epiactis prolifera* (Dunn, 1975) and *Urticina crassicornis* (Wedi and Dunn, 1983). In *Urticina* the development of the testicular cysts was found to be much more closely synchronized, as was found in the present study.

The lack of synchrony in the *A. fragacea* female gonad is partly due to the prolonged period of oocyte entry into the mesogloea. Not only does this generate asynchrony of itself, but it may mean that different oocytes may begin growth and vitellogenesis under very different conditions. Not only may external conditions, such as temperature and food availability, vary over this period, but the internal environment within the gonad may also change. Small oocytes entering the mesogloea

early, during May and June, may have to compete for space and available nutrients with larger oocytes from the previous year. Those entering during periods of large-scale oocyte resorption may, on the other hand, find an ample nutrient supply. Thus growth rates after entry into the mesogloea may vary for different oocytes.

The reproductive cycles of a number of anthozoans have been investigated in recent years. These species come from a range of habitats and exhibit widely differing reproductive strategies, but most show an annual gametogenic cycle, and many show similarities to *A. fragacea* in other respects. In *A. fragacea*, the period required for oocyte growth appears longer than that needed for sperm development, and a similar situation has been reported in several corals. In the solitary coral *Balanophillia elegans*, oocytes are thought to take over two years to grow to maturity, while male development is complete in less than one year (Fadlallah and Pearse, 1982a). In *Paracyathus stearnsii*, oocyte growth takes 13-15 months, while sperm develop in nine months (Fadlallah and Pearse, 1982b). In *Paraerythropodium fulvum fulvum*, female development takes 10-11 months, the male only 7-9 months (Benayahu and Loya, 1983). *Xenia macrospiculata* shows a prolonged period of male and female gonad initiation similar to that shown by the *A. fragacea* female (Benayahu and Loya, 1984).

While the present findings on gametogenic cycles in *A. fragcea* seem broadly consistent with those for several other anthozoan species, they do not correlate well with those of Rostron (1970) and Chia and Rostron (1970) for the closely related *Actinia equina*. They report that, in this species, the growth of oocytes from 25 μm to their maximum

size takes only one month, and that male gonads also take only a similar period to develop. It is surprising that, when the time taken for oocyte development in most other anthozoan species studied varies between nine months and two years or more, in *A. equina* it should take only four weeks. Carter and Funnell (1980) studied field and laboratory populations of both *A. equina* and *A. fragacea*, and concluded that both showed annual gametogenic cycles. The population of *A. equina* sampled as part of the present study (see Chapter 10) also appeared to show an annual cycle. It is difficult to see how these findings can be reconciled with those of Chia and Rostron.

After a detailed examination of the relevant sections of Rostron's thesis, it seems possible that some of her results could be interpreted differently. She plotted three graphs (Text Figs. 2, 3, and 4; pages 56-59) showing the way in which the proportion of adults bearing gonads, the diameter of the testicular cysts and the diameter of oocytes varied during the year. On a number of occasions during the year, all three graphs fall to zero simultaneously, and on several other occasions either testicular cyst diameter or oocyte diameter is recorded as zero. All these zero points were interpreted as marking the end points of gametogenic cyles, which were therefore thought to be of very short duration. However, it seems at least possible that the failure to find gonads or gametes on particular occasions was simply due to insufficient sample size, since, as was also found by Carter and Thorpe (1981), only a small proportion of even large *A. equina* individuals possess gonads. If the zero points are interpreted as being due to sampling error and disregarded,

the resulting graphs are quite consistent with an annual gametogenic cycle. The subsequent paper (Chia and Rostron, 1970) reports findings from two separate populations of *A. equina*, which were sampled independently. One population was at Broadstairs in Kent (presumably sampled by Rostron) while the other was at Cullercoats in Northumberland (presumably sampled by Chia). However, only the Kent population was examined histologically, so data for gonad development and cycles must have come from only one population, and may have been based solely on Rostron's thesis work. Thus it seems possible that an error of interpretation in Rostron's thesis could have been carried over into the joint paper. If the above argument has any validity, it could go some way towards explaining the discrepancies between Chia and Rostron's findings and those of other studies, including the present one.

The observation that spawning in *A. fragacea* occurs during the months of June and July raises the possibility that temperature is an important factor in regulating the gametogenic cycle. Jennison (1978, 1979) noted that in the anemone *Anthopleura elegantissima*, gamete development was correlated with rising temperature, and that spawning occurred during peak summer temperatures. He obtained further evidence that temperature was important by studying anemones living near a warm water outfall from an electricity generating station. Water temperatures here were up to $10^{o}C$ higher than at a nearby control site. He found that male gonads developed more rapidly and male and female spawning occurred earlier in anemones near the outfall. Animals transplanted from the control site to the outfall underwent greatly accelerated

gametogenesis and spawned up to five months earlier than those left at the control site. However, for an intertidal animal subjected to wide short-term temperature fluctuations, other factors such as photoperiod, may also be involved in the regulation of the cycle.

The present study suggests that *A. fragacea* is dioecious, with a sex ratio close to unity. The Shaldon population samples, however, showed a preponderance of males. These results, however, may give only a poor indication of the state of the population as a whole. Sample sizes were small, and animals were not collected by a rigorous random sampling procedure. Additionally, only a limited number of gonads were examined from each animal. Sampling was geared towards obtaining gonad material for cytological investigation, and caution is needed in extrapolating findings to the general population.

Chapter 4

THE EARLIEST STAGES OF OOGENESIS

INTRODUCTION

There appears to be no consistent pattern relating to the origin of the germ cells among coelenterates. Miller (1983) has recently reviewed the relevant literature, which indicated that in different species the germ cells may derive from ectodermal or endodermal cells. In some species, e.g the hydrozoan *Podocoryne carnea*, the male and female germ cells apparently arise in different layers, and several instances of migration from one cell layer to the other during germ cell development have been reported. In *Hydra* species, some of which have been extensively studied, the gonads are located in the ectoderm, and the germ cells derive from ectodermal interstitial cells. The gonads of sea anemones are endodermal, and several recent authors have indicated that the germ cells derive from endodermal interstitial cells (Dunn, 1975; Jennison, 1979; Szmant-Froelich *et al*, 1980). However, these authors have employed only light microscopy, and the early germ cells are difficult to distinguish clearly at this level. Miller (1983) concludes that the origin of scyphozoan and anthozoan germ cells has not been studied in detail in any case.

During the present study with the electron microscope, numerous small cells were found among the endodermal gonad epithelial cells at various times of year. On the basis of their ultrastructure and the timing of their occurrence with respect to the annual gametogenic cycle described in Chapter 3, these cells have been grouped into three categories. These three classes of cells will be described, initially without comment on their status. A consideration of the relative status of the categories will be given in the Discussion section of this chapter.

RESULTS

In samples of gonads taken between May and July, large numbers of small cells (5 - 12 μm in diameter) with relatively large nuclei and densely staining cytoplasm were found among the bases of the gonad epithelial cells, as were larger (up to 25 μm) oocytes. Similar small cells were subsequently found in samples from other times of the year, but in much reduced numbers.

These small cells are not easily distinguished in light microscope sections. On the basis of their size and ultrastructural appearance they can be divided into three broad categories, which have been termed Type I, II and III cells. The possible significance of the various types will be considered in the Discussion section at the end of the chapter.

1. Type I Cells

Type I cells were found scattered sparsely among the bases of the gonad epithelial cells during the period between June and October. They were found in very much greater numbers in the May samples. In some areas of the gonad at this time, especially near the junction of the gonad with the mesenteric retractor muscle, Type I cells were found in very high concentrations, and formed an almost continuous layer at the mesogloea/endoderm boundary. They are the smallest of the three cell types, and typical Type I cells are shown in Figs.4.1 and 4.2 They are 5 - 9 μm in diameter, and are usually round or slightly elongate.

a) Nucleus

The nucleus is very large relative to the size of the cell, up to 4 - 6 μm in diameter, and is of generally very low electron density, especially when compared to the densely staining cytoplasm. The chromatin is usually seen as irregular electron dense masses separated by areas of low electron density. These in turn consist of a finely filamentous material arranged in a loose reticulum in an electron lucent matrix. A single nucleolus is often seen (Fig.4.3), consisting of a coarsely granular material, always closely appressed to the nuclear envelope and often associated with the dense chromatin areas.

Small granules of dense material are usually found in nuclei of cells of this type, and have been termed nuclear granules (Fig.4.4). They are roughly spherical and vary in size between 40 and 100 nm. They appear to be randomly scattered through the nucleus. Rarely, nuclei are seen to contain one or more vesicles, up to 600 nm in diameter, full of small dense particles (Fig.4.2). These vesicles are bounded by a thin dense layer which does not show a tri-laminar unit membrane structure. It appears to link up with fibrils of the less dense fibrillar nuclear material. The enclosed particles appear spherical and are some 20 nm in diameter. They resemble β-glycogen particles, but their true nature is uncertain.

In the nuclei of many cells of this type, characteristic three-layered synaptinemal complexes can be seen (Fig. 4.4). These consist of two outer dense granular areas lying either side of a less dense, more finely granular central lamina.

The outer areas are separated from the central lamina by a gap of some 20 nm. Only rather short lengths of synaptinemal complex have been found. Sometimes thread-like structures resembling synaptinemal complexes but seeming less precisely arranged are seen (Figs. 4.5 and 4.6).

The nucleus is bounded by a well-defined nuclear envelope containing occasional nuclear pores. The dense chromatin areas often make contact with the inner surface of the nuclear envelope, and appear to attach to it by the formation of dense plaques, which may be widespread (Fig. 4.2) or tightly localized (Fig.4.7). Synaptinemal complexes and related structures may also contact the envelope (Fig. 4.6).

b) Cytoplasm

The cytoplasm appears dense and contains numerous free ribosomes but rather few other organelles. The nucleus is often located eccentrically in the cytoplasm, with most of the organelles contained in a cytoplasmic lobe on one side of the nucleus. The cytoplasm is normally seen to contain a number of mitochondria, a Golgi complex, glycogen deposits, lipid droplets, occasional dense bodies, small vesicles of various kinds and a flagellar basal-body-rootlet complex.

The mitochondria are small and usually squat, averaging about 400 nm in diameter and 700 nm in length in section. Occasionally elongate mitochondria of similar diameter but up to 1.5 μm long are observed. The mitochondrial matrix is generally homogeneous and electron dense. The cristae are numerous and in some cases are arranged in a characteristic lattice-like pattern (Fig.4.8). The mitochondria are usually clustered to form one or two loose groups rather than

distributed evenly throughout the cytoplasm.

The Golgi complex is usually situated close to the nuclear envelope, and consists of a stack of 5 - 7 parallel flattened cisternae containing a moderately electron dense material (Fig. 4.2). The density of this material often increases from one side of the stack to the other. The stack is often associated with a concentration of small vesicles and short membranous elements, all containing material of similar density and appearance to that found in the cisternae. Only a single Golgi complex was found per cell in section.

Glycogen is found in small deposits scattered throughout the cytoplasm, often associated with lipid droplets (Figs 4.7; 4.8). The smaller deposits contain glycogen mainly in the β-conformation, with an average particle size of 20 nm. Larger deposits may include a few typical rosettes of glycogen in the α-conformation, between 70 and 120 nm in diameter. The glycogen particles in these cells are always smaller than those found in the bases of the surrounding epithelial cells.

There are often one or more lipid droplets in the cytoplasm of Type I cells (Figs 4.8 and 4.9). These droplets are roughly spherical and range between 0.6 and 1.2 μm in diameter. They appear homogeneous and of low electron density. They are similar in appearance to, although usually smaller than, the droplets found abundantly in the bases of the gonad epithelial cells.

Various small, membrane bound vesicles are frequently found. The largest may be 500 nm in diameter and contain a

homogeneous electron dense material. Rarely, dense vesicles of this type contain small, lipid-like inclusions. The most numerous vesicles are smaller, some 150 nm in diameter, and enclose an electron lucid interior. Some of these vesicles also contain a single membrane bound core of moderately dense material.

Most Type I cells appear to possess a single flagellum (Figs. 4.1and 4.3). The flagellum arises from a basal-body-rootlet complex, which consists of two centrioles at right angles to each other, connected to a striated ciliary rootlet, extending into the cell. The complex appears identical to those found in the gonad epithelial cells as described in Chapter 3. Microtubules can often be seen radiating from the centrioles of the complex. In Type I cells, the complex is usually situated at the base of a deep invagination of the cell surface, so that for the first part of its length the flagellum is surrounded by a collar of cytoplasm (Fig. 4.3). Near its base, the flagellum has a complex structure (Fig. 4.4) identical to that described for gonad epithelial cells (see Chapter 3).

Many Type I cells are seen to contain one or two characteristic aggregations of finely granular dense material. These often consist of closely packed short cylinders of dense material, each cylinder being some 200 nm in diameter. As many as twenty such cylinders have been found conjoined in a single cluster, although between three and six is more common (Fig. 4.8). Sometimes each cylinder may enclose a central area of lower electron density. The clusters are often found in close association with

mitochondria, and may be found close to the nuclear envelope. However, they have not been observed to make actual contact with these or other organelles. Similar structres are found in later stages, and are discussed in more detail in Chapter 6, Section G.

Many Type I cells are seen to make contact with the mesogloea of the gonad. Many lie at the mesogloea/endoderm border and contact the mesogloea over a broad area (Fig. 4.2), while others are situated deeper in the endoderm and contact the mesogloea by means of a slender cytplasmic process (Fig. 4.9). Other cells which do not appear to contact the mesogloea may of course do so via a slender process out of the plane of section.

2. Type II Cells

Type II cells were found among the bases of the gonad epithelial cells at all times of the year, but were more common between June and October. In June and July, they were found in large numbers close to the gonad/retractor muscle boundary.

These cells are similar in many respects to the Type I cells, and only the points of difference between them will be described in detail. They are rather larger than Type I cells, and may reach some 10-11 μm in diameter. Type II cells tend to be more variable in shape and irregular in outline than Type I cells. They may be spherical or quite markedly elongate, in which case their longest dimension may reach 13-15 μm. Their greater size is largely the result of a greater volume of cytoplasm, although the nucleus may also be slightly larger, and may measure 6-9 μm in diameter.

The other most noticeable difference from Type I cells is the appearance of the nucleus. The nuclear material tends to be of greater overall electron density, and is more uniform in density and appearance (Figs. 4.10, 4.11). Thus the greater part of the nucleus contains a uniform finely granular material of moderate electron density. Areas of denser, more coarsely granular chromatin material are found scattered throughout the nucleus, and one or rarely two nucleoli are seen, always situated close to the nuclear envelope. Denser nuclear granules, up to 100 nm in diameter, are found in greater numbers in Type II cells than in Type I cells. The well-defined nuclear envelope contains rather more nuclear pores than are found in Type I cells. Synaptinemal complexes are occasionally found in Type II cells (Fig. 4.10), again as isolated short lengths, and sometimes making contact with the nuclear envelope.

The cytoplasm is more extensive and more richly endowed with organelles and inclusions than in Type I cells. In particular, the number of mitochondria and tha amounts of glycogen and lipid are significatnly increased. The mitochondria are similar in appearance to those of Type I cells, and again tend to be arranged in one or more groups. The individual mitochondria may tend to be smaller, but the groups may contain many more mitochondria, of which a higher proportion may be elongate. Aggregations of rings of dense material with a characteristic honeycomb appearance are again commonly found associated with the mitochondrial groups.

The glycogen tends to be found localized into fewer, larger deposits within the cytoplasm, and more is found as rosettes of the α-conformation. Glycogen deposits are

often associated with lipid droplets and dense bodies of various kinds. Membrane-bound dense bodies are more common than in Type I cells (Figs. 4.11, 4.12) and small amounts of endoplasmic reticulum may be found scattered randomly through the cytoplasm.

3. Type III Cells

Type III cells were found less commonly than either of the preceding cell types. They may occur occasionally at any time of the year, but were only found in any numbers in the May samples, and then only in areas containing large numbers of Type I cells.

Type III cells average some 12 μm in diameter, and are in many ways similar to Type II cells, but are distinguished from them largely on the basis of their nuclear appearance. The Type III cell nucleus is large relative to the size of the cell, but is of generally very low electron density (Fig. 4.13). Irregular, dense chromatin areas are separated by areas of very low electron density, in a manner reminiscent of the smaller Type I cells. In Type III cells, however, the nuclear envelope is always poorly defined, very often incomplete, and sometimes apparently missing altogether. Large, seemingly empty vacuoles are often seen around the periphery of the nuclear area (Figs. 4.14 and 4.15).

The cytoplasm may be quite extensive, and is similar in general appearance and organelle complement to that of Type II cells. Mitochondria may be numerous (Fig. 4.13), but are usually small. A basal-body-rootlet complex may be found, but endoplasmic reticulum has not been seen in these cells.

DISCUSSION

For many years it has been known that in sea anemones the gametogenic cells arise in the epithelia of the mesenteries and then migrate into the mesogloea where subsequent gamete maturation takes place (Hyman, 1940). Recent light microscope studies of sea anemone gametogenesis have confirmed this view. Dunn (1975) described how oocytes in *Epiactis prolifera* were derived from small cells with relatively huge nuclei which arose and proliferated in the endodermal epithelial layers and migrated into the mesogloea when they reached a diameter of 20 - 25 μm. Similarly, Jennison (1979) for *Anthopleura elegantissima* described cells with large nuclei and little cytoplasm within the endoderm of the gonad, which grow and come to line the mesogloea/endoderm border before migrating into the mesogloea. However, the cellular origins of these cells within the endoderm is far from clear. On the one hand, this reflects the lack of detailed information available for the early stages of gametogenesis in anthozoans, when the cells are small and difficult to resolve with the light microscope and until this study have not been examined by electron microscopy. However, it also reflects a deeper lack of understanding of the basic cellular organization and dynamics of anthozoan coelenterates.

In *Hydra* and at least some other hydrozoans, the gametes are known to be derived from undifferentiated cells known as interstitial cells (Bode and David, 1978; David, 1983). Most previous authors on anthozoan gametogenesis have also indicated that the gametes are derived from

endodermal interstitial cells (Hyman, 1940; Dunn, 1975; Jennison, 1979; Szmant-Froelich *et al*, 1980). However, there appears to be little hard evidence to support this view, other than the small size and unspecialized appearance of the early germ cells, and by analogy with *Hydra*. The development of the interstitial cell theory is central to modern coelenterate biology and so will be very briefly reviewed here. The existence of small, rounded, apparently undifferentiated cells among the larger epithelial cells in *Hydra* has been known for many years (see for example Stolte, 1936), and the so-called 'I-cell theory' gained prominence during the period 1930 - 1970. The theory largely rests on the finding that mitoses are very rarely seen in *Hydra* epithelial cells, such that it was thought that epithelial cell mitosis could not be sufficient to maintain the growth and budding activities of the animal. Interstitial cells, however, occurred in groups and were thought to be a rapidly dividing cell population. It was therefore proposed that the interstitial cells could differentiate into more specialized cell types, and so constituted and 'embryonic reserve' of mitotically active small cells which could produce a variety of other cell types as required (Brien, 1953). Using the electron microscope, Lentz and co-workers during the 1960's (Lentz, 1966), showed interstitial cells differentiating into a range of epithelial cell types, nerve cells and nematocytes. Interstitial cells were thus thought to be central to the regeneration, budding and gametogenic activities of the animal.

In the early 1970's, *Hydra* cell and developmental biology entered a new era of rapid advancement, aided by the introduction of a number of techniques. Foremost among these was the method of mass culture of asexually reproducing *Hydra* developed by Lenhoff and Brown, (1970), by which huge numbers of genetically similar *Hydra* individuals could be reared under controlled conditions. Another major advance was the maceration technique of David *et al* (1973), by which the individual cells of the *Hydra* could be separated while retaining their characteristic morphology. In this way the precise cellular composition of different body regions could be established and quantified, both during normal development and in experimental situations. Campbell (1973), using autoradiography and cell marking techniques, was able to elucidate the cell and tissue migrations which occur along the *Hydra* body column. The mass of new information emerging was not all consistent with the earlier findings. It now seems that interstitial cells may have a more restricted developmental repertoire than previously thought. In the steady state, asexual animal, probably only nerve cells and nematocytes are derived from interstitial cells, while the epithelial cells constitute self renewing populations. The capabilities of the epithelial cells have been strikingly demonstrated by Marcum and Campbell (1978). They have succeeded in producing and maintaining populations of *Hydra* containing no interstitial cells whatsoever. These animals also lack nematocysts and nerves, and so are deficient in many respects. However, if they are cultured carefully, they continue to

grow and show budding and regeneration ability. Contrary to the assertion in Nieuwkoop and Satasurya (1981), these so-called 'epithelial' *Hydra* can be maintained indefinitely and do not necessarily degenerate and ultimately die.

David and Murphy (1977) have shown that the interstitial cells are truly pluripotent, in that a clone of cells, derived from a single cell, can produce both nerve cells and nematocytes. Most recent work has been carried out using asexually reproducing *Hydra*; the mechanism of sexual reproduction has received less attention. However, it seems clear that the gametes are also derived from interstitial cells. It was thought that germ cells were produced from the same stock of interstitial cells which produce nerves and nematocytes in asexual *Hydra*, but very recently, preliminary evidence has emerged that a sub-population of interstitial cells destined only for germ cell differentiation may exist (Littlefield, 1984). It is interesting to note that recent papers from the American, Japanese and German groups most actively involved in modern *Hydra* research usually make no mention of the earlier work showing interstitial cell differentiation into epithelial cell types. Equally, recent reviewers from outside these groups (Niewkoop and Satasurya, 1981; Van der Vyver, 1982) have understressed the recent work and still suggest a wide-ranging role for the interstitial cell, as the agent primarily responsible for cell replacement, budding and regeneration.

If the situation in *Hydra* is still controversial, that among the Anthozoa is one of almost complete ignorance.

Nothing is known about epithelial cell migration in anthozoans. Loss of nematocytes must occur as a consequence of feeding, but the mechanism of nematocyte replacement is not understood. Most fundamentally, it is still not clear whether adult anthozoans possess interstitial cells. Westfall (1966) has described nematocyte differentiation from interstitial cells in small *Metridium senile* individuals but it is not clear whether these cells can differentiate into other cell types. Singer (1971) found cells which he termed interstitial cells in regenerating *Aiptasia diaphana*, but their role has never been elucidated. Several authors have suggested that interstitial cells do not occur in Anthozoa. Bouillon (1968), in his description of cell types in anthozoans, makes no mention of interstitial cells. Chapman (1974) points to amoebocytes as possibly being functionally equivalent to hydrozoan interstitial cells. Van Praet (1974) and Van Praet and Doumenc (1975) did not find interstitial cells in regenerating *Actinia equina*, and concluded that de-differentiation of epithelial cells was the major source of new cells for regeneration. In the present study, no cells which could be reliably identified as interstitial cells were encountered, either in the gonads and mesenteries or in any other body regions examined. Profiles of small cells with relatively large nuclei were occasionally seen, but epithelial cells sectioned transversely can generate such profiles, and the complex nature of fixed sea anemone epithelia calls for care in the interpretation of such images. Perhaps the most potentially fruitful approach to the problem of interstitial cells in

Anthozoa was that adopted by Minasian (1980). He used tritiated thymidine autoradiography to investigate the incidence of mitosis in intact, adult individuals of the sea anemone *Haliplanella luciae*. He found mitoses in both endoderm and ectoderm of all regions of the body. Most epithelial cells have apically located nuclei, and many of these were labelled, but he also found labelled areas in the basal layers of the epithelia. Some of these could represent mitotically active amoebocytes, but could also be taken as evidence for the existence of interstitial cells in the basal interstices of the epithelia. Unfortunately, with the light microscope wax sections he employed, individual cells and cell types could not be distinguished clearly. It is a matter of some urgency to repeat and extend Minasian's work, using light microscope autoradiography of thin plastic sections and then electron microscope autoradiography to catalogue the cell types present in different regions of the anemone, and assess their mitotic activity. Research along these lines might clear a major obstacle in our understanding of sea anemone cell biology and tissue dynamics.

In the present study it was considered prudent not to prejudge the status of the small cells found in *A. fragacea* gonads at the beginning of the gametogenic cycle. The categories which could be distinguished on morphological grounds were therefore merely designated Types I, II and III cells. Some tentative conclusions as to their status can, however, be attempted.

The nuclei of Type I and Type II cells were frequently found to contain synaptinemal complexes. These structures have been reported from the germ cells of a great many species (see, for example, Westergaard and Von Wettstein, 1972), and are thought to represent structures by which homologous chromosomes become aligned and pair at synapsis. They are therefore taken to be indicators of cells in the zygotene or pachytene stages of meiotic prophase. Similar synaptinemal complexes have also been found in anthozoan spermatocytes (Schmidt and Zissler, 1979), and have been found in male germ cells in *A. fragacea* (see Chapter 12). Type I cells appeared in the gonad in spring, and had disappeared by early summer. Type II cells appeared slightly later than Type I, and their numbers fell throughout the summer. It is therefore suggested that Type I cells represent an early, and Type II cells a slightly later, stage in the development of the female germ cells. The finding of synaptinemal complexes suggests that at least some (and possibly all) of these cells are undergoing meiosis and so are already oocytes, rather than oogonia or any other cell type. No signs of mitotic activity were found in any Type I or II cells, and it would appear that, in *A. fragacea*, a coherent series of developing cell types can be traced, starting from Type I cells, leading to Type II cells and on to larger oocytes which enter the mesogloea and begin vitellogenesis, with no evidence of intervening cell divisions. This series will be further illustrated in the following chapter.

While the evidence strongly points to Type I and II cells being early oocytes, the status of the Type III cells is less clear. Many Type III cells simply appear degenerate; the most straightforward explanation is that they represent a proportion of germ cells which break down at an early stage and are lost. Type III cells were found to be most numerous in areas of gonad containing large numbers of very early oocytes. The possibility exists that these cells are indicative of an oogonial or proliferative phase in oocyte production. The clumped appearance of the chromatin in these cells, and their lack of a well-defined nuclear envelope might be suggestive of mitotic activity, or of a failure of mitosis to proceed normally. However, no really clear evidence of mitosis was obtained. Type III cells were encountered rarely in comparison with the other cell types, and no firm conclusion can be reached as to their significance.

There has been no previous detailed ultrastructural study of other anthozoan oocytes at a stage early enough for useful comparisons to be drawn with the *A. fragacea* oocytes described here. However, there have been several published studies of early oocytes from among the Hydrozoa. In particular, Kessel (1968a) described all stages of oogenesis from an unidentified trachyline medusa, and Aizenshtadt (1974) and Noda and Kanai (1977) have described the formation of oocytes from interstitial cells in *Hydra*. The earliest oocytes of *A. fragacea* seem to be generally similar in appearance to the corresponding cells in these hydrozoans. All show features usually taken to be

characteristic of cells at a low level of differentiation - a large nucleus relative to the size of the cell, scant basophilic cytoplasm with numerous ribosomes and little organized membrane systems. Some Type I cells in *A. fragacea* seem to have relatively even less cytoplasm than these other cells, including the interstitial cells described by Noda and Kanai (1977). They do tend to contain more glycogen and lipid drops than the others, however.

The arrangement of mitochondria in groups is common to all these types of cell. The mitochondria in *A. fragacea* seem more compact and have a more electron dense matrix than those seen in *Hydra* or described by Kessel. The unusual lattice-like arrangement of cristae has not been remarked by other authors and will be discussed further in Chapter 6. The honeycomb arrangement of conjoined cylinders of dense material has also been found in larger *A. fragacea* oocytes, and in early male germ cells (see Chapter 11). It may represent some form of nuage material, and its possible significance will be discussed in Chapter 6. Kessel also reports the presence of basal-body-rootlet complexes in his early oocytes. They tend to be associated with microtubules, and are generally very similar to those described here. Kessel does not mention flagella arising from these complexes however. In early *A. fragacea* oocytes, these complexes often give rise to flagella, which usually arise at the base of a deep invagination of the cell membrane. This arrangement is similar to the cytoplasmic collar found around the anterior portion of the tail flagellum in many anthozoan sperm (Hinsch, 1974; Hinsch and

Clark, 1973), including those of *Actinia* (see Chapter 12). Basal body rootlet complexes have also been found in other non-epithelial cell types in *A. fragacea* as well as in the flagellated epithelial cells. Granular amoebocytes, the mesogloeal cells of the upper filament and early male germ cells all have these complexes, which presumably cannot serve as functional flagellar basal apparatuses in the enclosed situations in which these cells are found. Chapman and James (1973) cite numerous reports of 'submerged' cilia from vertebrate and invertebrate tissues, and conclude that in most cases there is probably no functional significance to most of these cilia, but that their inappropriate formation is easily triggered in many cells. They cite Rash *et al* (1969) as saying that most primitive or differentiating cells have a cilium during interphase. Schmidt and Holtken (1980), however, state that anthozoan female germ cells do not possess such a complex, a view with which the present findings are not consistent.

The apparent absence of an obvious oogonial proliferative phase in *A. fragacea* is of interest. No unequivocal signs of mitosis were seen among any of the cell types observed here, although some Type III cells could possibly be interpreted as having recently undergone mitosis. Obvious precursor stages to the Type I cells could not be detected in earlier gonad samples. It may be that mitosis occurs very rapidly and so is rarely seen in random sections, as has been suggested by Doumenc (1977). Alternatively, mitosis could be restricted to localized areas of the gonad with subsequent migration of the daughter

cells, or could be restricted to a brief period of the day or night. Finally the oocytes could be produced directly with no intervening proliferative phase. Interestingly, I have observed mitoses among early female germ cells in *Cereus pedunculatus*, in spite of a much less intensive study of that species, see Chapter 10.

Thus the problem of the origin of the germ cells is still unresolved. While the early germ cells are morphologically similar to hydrozoan interstitial cells, there does not appear to be a resident population of such cells in the gonad from which they could arise. Migration into the gonad from a distant site is another possibility. They could derive from epithelial cells, either by de-differentiation or by unequal cell division, as has been proposed for the formation of interstitial cells in *Hydra* (Brien, 1954; Tardent, 1954; Brien and Reniers-Decoen, 1955) although not in more recent studies. The problem of the origin of the germ cells will be returned to in the chapter dealing with nuage material (Chapter 6, Section G).

Chapter 5

OOCYTE GROWTH WITHIN THE ENDODERM AND ENTRY INTO THE MESOGLOEA

INTRODUCTION

Although it has been known for many years that, in sea anemones, the female germ cells migrate from the gonad epithelium into the mesogloea during their development (e.g Hyman, 1940), the process of entry into the mesogloea has not been studied in detail. In a major study of gametogenesis in the sea anemone *Actinia equina*, Chia and Rostron (1970; also Rostron, 1970) do not mention an endodermal phase or the entry of oocytes into the gonad mesogloea. The process of entry has been illustrated at the light microscope level for the anemone *Peachia quinquecapitata* (Spaulding, 1974), and described from *Epiactis prolifera* (Dunn, 1975) and *Anthopleura elegantissima* (Jennison, 1979), but few details are given. It has been mentioned in the ultrastructural studes of Schmidt and Schafer (1980; Schafer and Schmidt, 1980; Schafer, 1983), but has not been illustrated or described in detail. The previous chapter discussed the possible origins of the germ cells, and described the earliest identifiable oocytes. This chapter considers ultrastructural aspects of the phase of oocyte development within the endodermal epithelium, and the subsequent entry of oocytes into the gonad mesogloea.

RESULTS

1. Growth Within the Endoderm

As suggested in the previous chapter, oocytes arise in the endoderm of the gonad, principally during spring and early summer. These cells undergo a variable period of growth within the endoderm before entering the mesogloea. Oocytes enter the mesogloea over a protracted period during summer and autumn, and the number of oocytes remaining in the endoderm decreases during this period. Thus oocytes, even within a single gonad, do not develop in synchrony, and the size at which oocytes enter the mesogloea also varies considerably. The major changes which the oocyte nucleus and cytoplasm undergo during this phase of oocyte growth are described below. A small proportion of oocytes begin vitellogenesis while within the endoderm; however, most only begin after entry into the mesogloea, and, for this reason, discussion of vitellogenesis will be delayed until the following chapter.

a) Nuclear changes

In the smallest recognizable oocytes, the nucleus is characteristically of very low electron density (see Chapter 4).

As the ooctyes enlarge, however, the density of the nucleus progressively increases. The irregular, dense chromatin areas initially become more extensive (Figs. 5.1-5.3) but later become smaller and less obvious (Fig.5.4). The nucleolus becomes more clearly defined and larger, and becomes spherical, finely granular and homogeneous (Fig.5.4). The smaller nuclear granules described in the previous chapter are common throughout the endodermal phase and are

usually randomly distributed within the nucleus. Nuclear fibrillar bodies may be found in endodermal oocytes greater than 15 μm in diameter, but they become more numerous during vitellogenesis and are discussed in Chapter 6, Section A.

Throughout oocyte growth, the nucleus is bounded by an intact, typical double membrane nuclear envelope, bearing nuclear pores. The pores appear to become more numerous and more closely packed as the nucleus enlarges. Synaptinemal complexes have not been observed in oocytes larger than 12 μm in diameter.

b) Cytoplasmic changes

The smallest oocytes contain relatively little cytoplasm. As the oocytes enlarge, the amount of cytoplasm and the numbers of organelles greatly increase, but the cytoplasm retains its generally unspecialized appearance until the onset of vitellogenesis.

Most endodermal oocytes are seen to contain one or more Golgi complexes. These resemble the complexes found in the smallest oocytes, except that, in larger oocytes, many are found associated with single cisternae of endoplasmic reticulum (Fig 5.5). The size, number and appearance of the Golgi complexes do not markedly change as the oocyte grows until it begins vitellogenesis. The smallest recognizable oocytes contain little or no endoplasmic reticulum. As they grow within the endoderm, most oocytes come to contain a small number of short lengths of randomly arranged ER, which may be studded with ribosomes (Fig 5.4). Major development of the ER begins with vitellogenesis (see Chapter 6, Section B.

Lipid droplets are very often found associated with accumulations of glycogen during the endodermal phase (Fig. Two types of lipid droplet can be distinguished. The more common type is 0.5 to 1.5 μm in diameter, homogeneous, and of low but perceptible electron density. The second type is similar in size, but appears completely empty, possibly as a result of extraction of the contents during processing. Large endodermal oocytes generally appear to contain relatively less stored lipid than smaller ones.

Flagellar basal-body-rootlet complexes are often found within endodermal oocytes (Fig 5.7). In very small oocytes (see Chapter 4), these complexes may give rise to flagella, but this has not been observed in oocytes larger than 12 μm. Complexes were found less frequently in larger oocytes, but this might simply reflect the lower probability of encountering a single complex in sections through the larger oocyte volume.

In localized regions of the oocyte, the cytoplasm may contain large numbers of membrane-bound vesicles (Figs 5.8,5.9). These range in size from 100 to 400 nm, averaging some 200 nm in diameter. Many of the vesicles appear empty, while others are wholly or partly filled with material of moderate electron density. Yet others may contain a smaller, membrane bound body which itself may contain granules of dense material resembling glycogen. Vesicles are often found near the oocyte surface, and become more numerous once the oocyte begins vitellogenesis.

Multivesicular bodies are occasionally found in endodermal oocytes. These are spherical, membrane bound sacs, usually about 1 μm in diameter, containing small vesicles,

each 40 to 50 nm in diameter and the occasional larger vesicle (Fig 5.3). They may occur anywhere in the cytoplasm of oocytes of any size, but were never found in large numbers.

Nuage material similar to that described in Chapter 4) may be found in endodermal oocytes of all sizes. In oocytes up to some 10 μm in diameter, it usually appears as conjoined cylinders of electron dense material (Fig 5.1). In some slightly larger oocytes, each cylinder may contain a cylindrical core made up of fine fibrils arranged longitudinally. In oocytes over 10 μm in diameter, the areas of nuage material tend to be more extensive but less precisely arranged. Many of the fibrillar cores may not be surrounded by dense material (Fig 5.3)and occasionally the cores may extend for 5 μm through the cytoplasm (Fig 5.10). The dense material may be present as small fragments or irregular masses rather than conjoined cylinders (Figs. 5.10, 5.11). Often more than one area of nuage material may be found in a single section through an oocyte, and these areas may be widely separated within the cell.

In oocytes larger than 10 μm in diameter, the nuage material is often associated with other structures. Very commonly, the fibrillar component is found next to or extending through deposits of glycogen (Figs. 5.12, 5.13). This association was encountered so frequently that it is not thought to be merely fortuitous, although its significance is unclear. Nuage also may be associated with areas of dense cytoplasm containing a finely granular material (Fig. 5.11). These dense areas are usually roughly circular in section, and may be 1 to 2 μm across. Nuage is also found

associated with groups of membranous tubules (Figs. 5.11, 5.13). The tubules average some 80 nm in diameter, and tend to be closely packed and roughly aligned rather than randomly arranged. Many of the tubules are curved such that circular profiles are often seen in section alongside longitudinal ones. Additionally, groups of bristle-coated membranous vesicles may be found in the vicinity of nuage material (Fig. 5.13).

c) Oocyte surface changes

While within the endoderm, the oocyte is surrounded by the basal processes of the endodermal epithelial cells. These processes vary in size and are often irregular in shape. Over most of its surface, the oocyte is separated from these processes by an irregular intercellular gap varying between 50 and 150 nm in width. Small, apparently empty membrane bound vesicles, 100-200 nm in diameter, which are common at this level in the endoderm, may be found in this gap. In localized areas the endodermal cell membrane closely follows the surface of the oocyte, with a narrow and rather constant inter-membrane gap of about 30 nm. In these regions, desmosome-like intercellular junctions are occasionally found between the oocyte and endodermal cell membrane (Fig 5.1). Apart from these junctions, the endoderm immediately around the oocyte shows no specializations or differences from the remainder of the endoderm. At no time while the oocyte is within the endoderm is an extracellular layer visible around the oocyte plasma membrane.

Once the oocytes reach about 12 μm in diameter,

cytospines start to appear on the oocyte surface (Fig. 5.4). Cytospines resemble large microvilli, and in small oocytes they are about 150 nm in diameter and contain a dense fibrillar core. The outer surface of the cytospine is covered with a fine glycocalyx-like material (Fig. 5.14). The cytospines usually lie folded flat against the surface of the oocyte. Initially the cytospines may form a single, fairly evenly spaced layer over regions of the oocyte surface, but as the oocyte enlarges, the groups of cytospines become more localized and discrete, and may be 2-4 cytospines deep (Fig 5.4). Among some groups of early cytospines, small bulges of the oocyte membrane are found containing material which closely resembles the contents of the cytospines (Fig 5.14). These bulges may represent points of insertion of cytospines into the oocyte membrane, or might be a stage in the formation of new cytospines.

Membrane bound vesicles, as described above, are often found near the surface of the oocyte (Fig5.14), especially near groups of cytospines. Depressions of the oocyte membrane which might be indicative of endocytosis are sometimes seen, but it is not certain that these vesicles are of endocytic origin.

2. Entry into the Mesogloea

After the period of growth among the basal processes of the endodermal epithelial cells, the oocytes enter the mesogloea of the gonad, where they will remain until spawning. There does not appear to be a specific size or stage of development at which oocytes enter the mesogloea. Some oocytes migrate into the mesogloea at a diameter of

about 12 μm, while others remain in the endoderm until they reach 40 μm and occasionally more. Since the development of oocytes even within a single gonad may be far from synchronous, some small oocytes may enter areas of mesogloea already densely populated by much larger oocytes.

Most oocytes appear to make their way into the mesogloea by a process resembling amoeboid movement. However, the possibility that the endodermal cell bases actively contribute to the movement of the oocytes cannot be ruled out. Prior to entry, some small oocytes already rest against the mesogloea, and others may contact it by means of slender cytoplasmic processes (see Chapter 4). For most oocytes, however, the first step of entry into the mesogloea appears to be to open a gap in the endodermal musculature. A short process is sent out from the surface of the oocyte which penetrates the musculature and makes contact with the mesogloea. This oocyte process does not appear to be specialised in any way. The process then pushes into the mesogloea, but in doing so it does not puncture the basal lamina which thus coats its tip (Fig 5.15). The oocyte now flows through the gap in the musculature and progressively passes into the mesogloea, carrying the basal lamina forward as it does so. As the portion of the oocyte within the mesogloea enlarges, the oocyte remains constricted at the point where it passes through the muscle layer, giving the oocyte a characteristic 'hourglass' or 'dumb-bell' shape which can be easily recognised in light microscope sections. With small oocytes, the nucleus is usually positioned near the leading edge of the cell, and so enters the mesogloea

relatively early (Figs. 5.16 and 5.17). With larger oocytes (above 15 μm in diameter), the nucleus usually occupies a more central position (Figs. 5.18 and 5.19).

When small (less than 15 μm) oocytes enter the mesogloea, their leading edge tends to be smooth and rounded (Fig 5.16). With larger oocytes, the initial process may project some distance into the mesogloea before it begins to fill out (Fig 5.18). When the process does fill out, its leading edge often bears projections which may facilitate further penetration of the mesogloea (Fig 5.17). Indeed, the larger the oocyte, the more irregular in outline does the portion within the mesogloea become (Fig 5.20). Not only may its leading edge be indented, but its lateral surfaces may be deeply invaginated such that regions of the oocyte appear discontinuous in section (Fig 5.21). Many of these invaginations may contain cytospines, and the constricted 'neck' region of the oocyte may appear vacuolated. The basal lamina covers the entire surface of the oocyte within the mesogloea, closely following all its irregularities, and can be seen to be continuous with the basal lamina of the neighbouring epithelial cells (Figs. 5.20 and 5.21).

When most of the oocyte has entered the mesogloea, it may become even more severely constricted at the muscle layer such that the portion remaining in the endoderm may be connected to the rest of the oocyte by only a slender neck (Fig 5.19). The constriction may be less severe than suggested by this micrograph since the section may not pass through the widest part of the neck. Oocytes can apparently remain with a small portion left in the endoderm for a

considerable time. The possible significance of this continued contact with the endoderm will be discussed below.

While it appears that most oocytes actively force their way into the mesogloea as described above, it is possible that in some instances the mesogloea may grow out around the oocyte, at least to some extent. Sometimes indications of mesogloeal outgrowth are seen around the oocyte process as it passes through the gap in the muscle layer (Fig. 5.22). Whether this is a result of new mesogloeal material being formed around the oocyte or whether existing material is pulled out of position as the oocyte enters is unclear. Some such mechanism may be important when large oocytes enter relatively narrow layers of mesogloea.

Occasionally 'spurs' or side brances extend out from the main layer of gonad mesogloea, and may make contact with oocytes in the endoderm (Fig.5.23). Whether such contacts are the result of directed mesogloeal outgrowth, directed oocyte migration or are merely fortuitous is uncertain. Some of the features of oocyte entry into the mesogloea are summarized in Diagram 6.

3. Maintenance of Oocyte/Endoderm Contact

Stages similar to that shown in Figure 5.19 in which most of the oocyte is within the mesogloea but a small portion remains in the endoderm, were encountered very frequently, suggesting that this situation might be relatively stable and long-lasting. A more detailed examination of oocytes at this stage revealed a number of

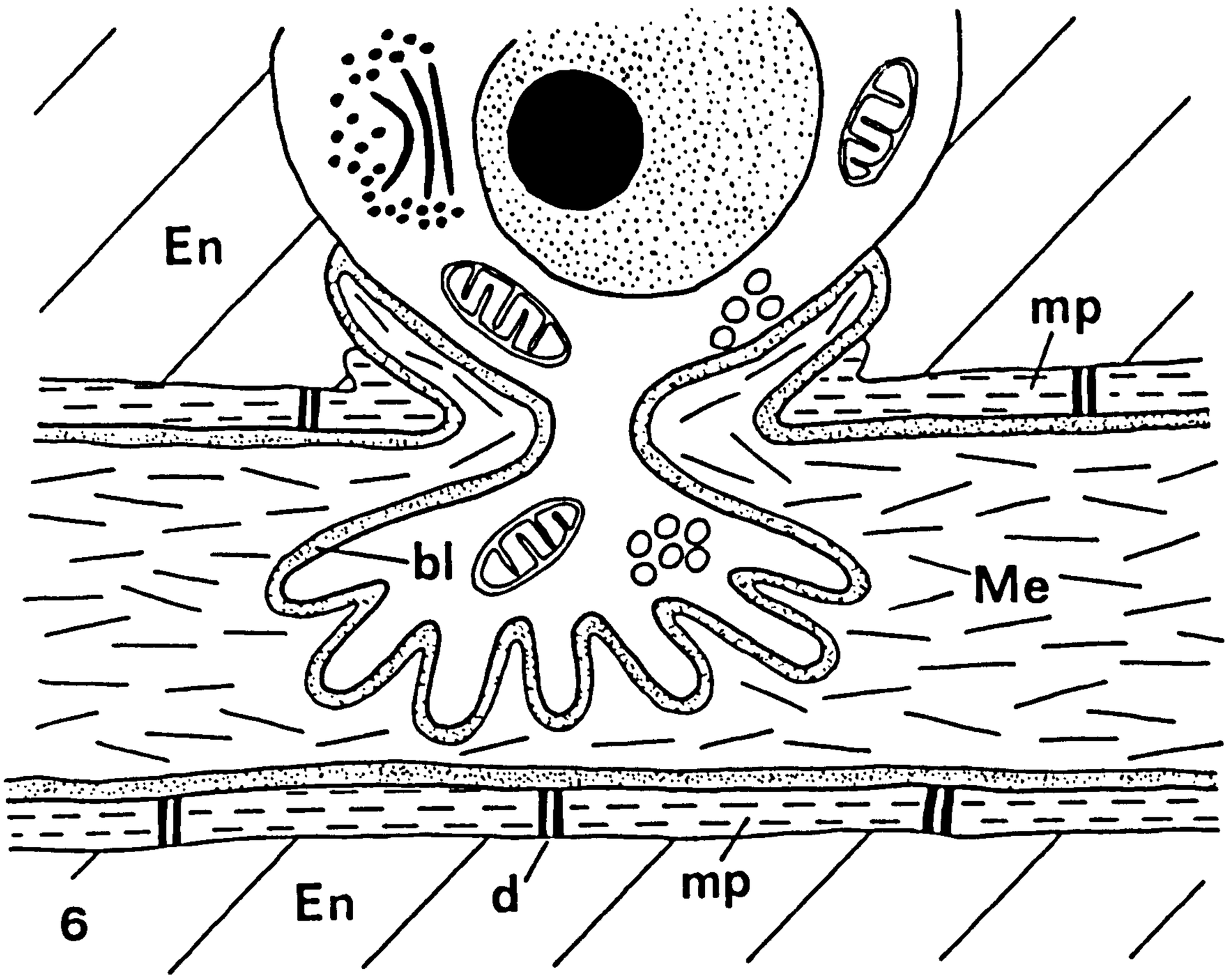


Diagram 6.

A summary diagram illustrating the entry of a relatively large (>15 µm diameter) oocyte into the mesogloea. The oocyte is constricted at the endoderm/mesogloea boundary, and its leading edge is deeply indented. The mesogloea is shown apparently growing or being pulled out around the neck of the oocyte, and the basal lamina covers the mesogloeal portion of the oocyte.

interesting features.

The small portion of oocyte remaining in the endoderm appears to enter into a close relationship with the bases of the endodermal cells surrounding it. Extensive intercellular junctions often form between the oocyte and the endodermal cell membranes (Fig 5.24). The neck region of the oocyte becomes indented, and endodermal cell bases and muscle processes are closely applied to its surface and follow its contours. The muscle processes often project out from the general line of the endoderm/mesogloea boundary as though they have grown or been dragged into the mesogloea by the entering oocyte. Again, desmosome-type junctions, similar to those found between adjacent muscle processes, link the muscle processes to the neck region of the oocyte.

Later in the formation of the oocyte/endoderm relationship, the surface of the endodermal portion of the oocyte may become densely covered with large cytospines (Fig 5.26) which project into and between the endodermal cell bases and form a region of intimate contact between oocyte and endoderm. The localization of the cytospines may be quite precise. In Figure 5.26 cytospines are virtually absent from the neck region of the oocyte, and from the region of contact with the muscle processes, where intercellular junctions form. However, they are large and closely packed where the oocyte makes contact with the unspecialised endoderm bases, which contain large amounts of lipid and glycogen.

A ring-shaped depression often forms around the base of the neck region in the surface of the mesogloeal portion

of the oocyte (Fig 5.25). This depression contains large numbers of cytospines, and cytoplasmic processes from the endodermal cells which have presumably grown or been pulled into the mesogloea with the oocyte. These processes may contain mitochondria, lipid droplets and often large quantities of glycogen (Fig 5.27). They may extend between the cytospines and contact the oocyte surface, and again may participate in desmosome formation. Away from the base of the depression, around the neck of the oocyte, the endodermal processes contain numerous microfilaments, usually aligned along the axis of the oocyte neck (Fig. 5.27).

The oocyte cytoplasm beneath areas of close contact between the oocyte surface and the endoderm contains large numbers of small, membrane bound vesicles (Figs. 5.26 and 5.27). These vesicles are similar in appearance to those found in the cytoplasm next to the region of oocyte/endoderm contact in the trophonema of larger oocytes (see Chapter 7). The main features of this stage of oocyte entry are summarized in Diagram 7.

Eventually the remaining endodermal portion of the oocyte is pulled into the mesogloea with the rest of the oocyte, and the ring-shaped depression in the surface of the oocyte becomes cup-shaped. However, contacts between the oocyte and the processes from the endodermal cells are not severed. These contacts are thought to play an important part in the formation of the trophonema (see Discussion) and hence in the nutrition of the growing oocyte.

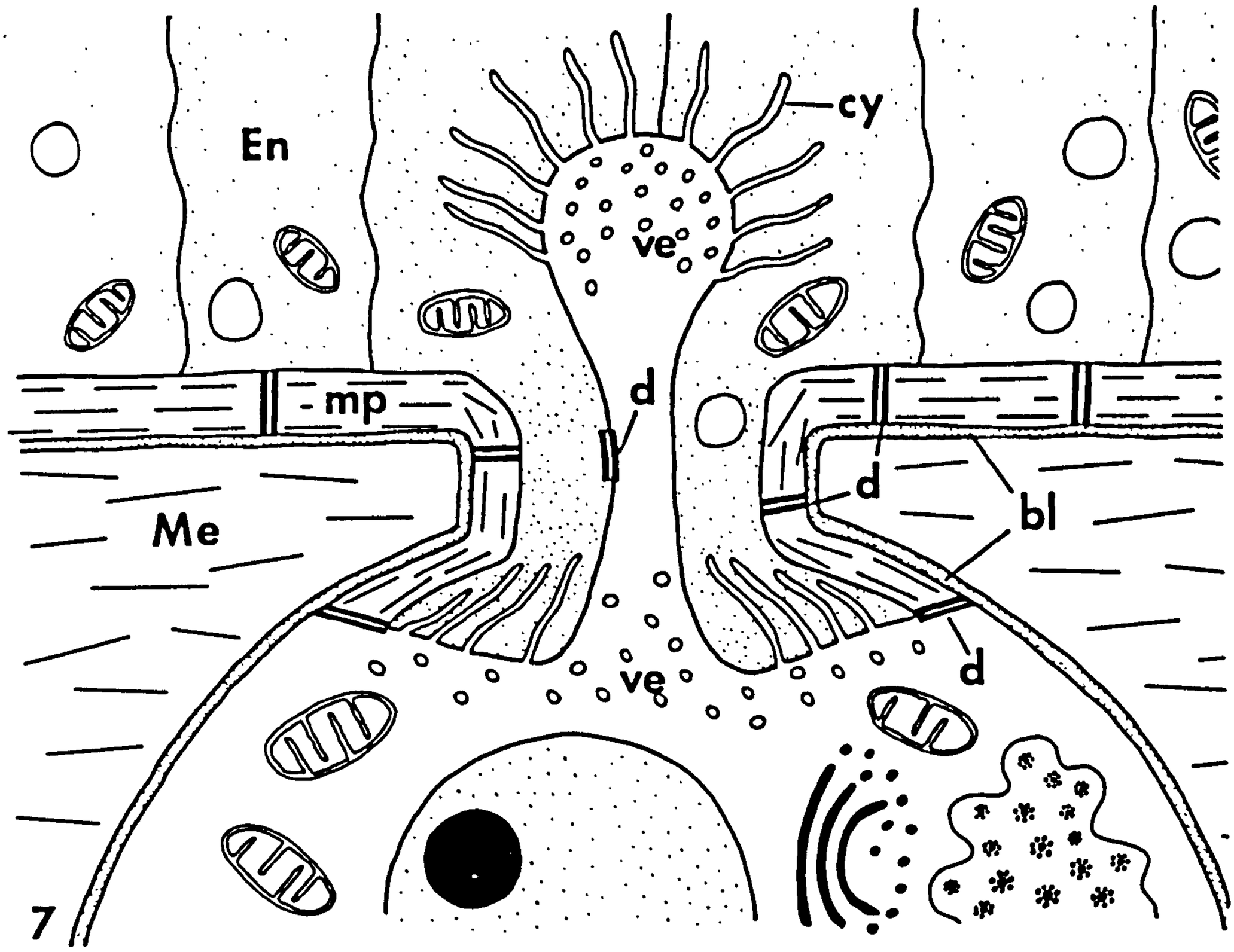


Diagram 7.

A summary diagram illustrating the relationship between oocyte and endoderm just before the completion of entry into the mesogloea. A small portion of the oocyte remains in the endodermal layer. Endodermal cell bases and muscle processes extend around the neck of the oocyte, and terminate in a ring-shaped depression in the mesogloeal portion of the oocyte.

DISCUSSION

The early, endodermal oocytes of *A. fragacea* share many characteristics with the pre-vitellogenic oocytes of many animal species (Norrevang, 1968; Anderson, 1974). In general appearance they resemble the early oocytes described by Schäfer and Schmidt (1980) and Schmidt and Schäfer (1980) from various anthozoan species. However, there appear to be a number of detail differences between early *A. fragacea* oocytes and the generalized descriptions given by Schmidt and Schäfer. The outline of the endodermal oocytes of *A. fragacea* is generally smooth with only small indentations, some of which may be concerned with pinocytosis. Their surface was not observed to be enlarged by manifold indentations containing glycogen-like material as well as many small vesicles as indicated by Schmidt and Schäfer. They also suggest that small endodermal oocytes are surrounded by many free vesicles and glycogen-like granules. In *A. fragacea* the oocytes are closely enveloped by the basal processes of the endodermal epithelial cells. These processes may contain lipid droplets and glycogen, and small vesicles are often found amongst them. However, these vesicles are not more numerous in the vicinity of small oocytes than elsewhere in the endoderm. Schmidt and Schäfer also state that there is a loose vitelline membrane of fine granular material on the surface of young oocytes still in the endoderm. In *A. fragacea* there is no extracellular layer of any description visible around the oocyte while it is within the endoderm. The oocyte plasma membrane

often contacts the membranes of the endodermal cell bases closely, with only a narrow inter-membrane gap, and desmosomes are sometimes formed between the two. As the oocytes enter the mesogloea, they do become surrounded by a layer of extracellular material. Whether or not this layer should be regarded as a vitelline membrane will be discussed below.

Schäfer and Schmidt (1980) suggest that ribosomes appear in the ooplasm during oocyte migration into the mesogloea. In *A. fragacea*, however, the ooplasm contains numerous ribosomes from the earliest recognizable stages (see Chapter 4). Kessel (1968a) found numerous free ribosomes in the early oocytes of a hydrozoan medusa, and their abundance is a characteristic feature of many unspecialized cells and primordial germ cells (Nieuwkoop and Satasurya, 1981).

The progressive increase in nuclear density seen in *A. fragacea* oocytes as they grow within the endoderm has not been remarked by other anthozoan workers. The nucleus increases in electron density until the phase of rapid nuclear enlargement which occurs early during vitellogenesis, after the oocyte has entered the mesogloea. The density of the nucleus then falls, and is generally low in all oocytes over about 50 μm in diameter (see Chapter 6, Section A).

The term cytospine was introduced by Dewel and Clark (1974) to clarify a confusing situation which arose when a structure previously described and named using the light microscope was observed by electron microscopy. Several earlier light microscope investigators had observed projections from the surface of anthozoan oocytes which

were generally referred to as spines (Gemmill, 1920; 1921; Chia and Rostron, 1970; Spaulding, 1972; Chia and Spaulding, 1972). With the electron microscope, Dewel and Clark (1974) found that in the sea anemone *Bunodosoma cavernata* the spines were actually bundles of smaller projections, each of which resembled a large microvillus. They proposed that each of the smaller projections be termed a cytospine, and their suggestion has been followed in the present paper. Cytospines have since been observed in many anthozoan species (Schmidt and Schäfer, 1980; Schroeder, 1982). The surface of the fully grown *A. fragacea* oocyte is covered by cytospines arranged in definite tufts (see Chapter 6, H) and the beginnings of this organization can be seen in larger endodermal oocytes. In large mesogloeal oocytes the fibrillar core is more obvious and projects from the base of the cytospine as a rootlet (Spaulding, 1974). Such rootlets were not observed in endodermal oocytes. Schmidt and Schäfer consider the cytospines to be resorptive organelles, and the present observations on small oocytes of *A. fragacea* are consistent with this function. Cytospines clearly increase the surface area of the oocyte, and may make close contact with the surrounding endodermal cells. Small vesicles, which may be pinocytic in origin, are more numerous in oocytes which bear cytospines and tend to be more numerous in the vicinity of groups of cytospines. Microvilli, which cytospines resemble, are thought to be involved in nutrient uptake in many other oocytes (e.g. Anderson, 1974; Schade and Shivers, 1980; Eckelbarger and Grassle, 1982). Cytospines may also be involved in the

formation of the trophonema (see below).

The clusters of electron dense cylinders and associated structures found in *A. fragacea* oocytes have also been found in early male germ cells (see Chapter 11) but as yet have not been seen in cells of any other type. This raises the possibility that these structures may correspond to the nuage material described from many germ cells (Eddy, 1975). Roosen-Runge (1977) describes nuage as "the dense fibrous material (order of magnitude 1 μm or less) seen in germ cells of many animals", a description which could be applied to these structures. Nuage material has not previously been reported from other sea anemone germ cells, but also occurs in *Cereus pedunculatus* oocytes (see Chapter10). Nuage has been reported in germ cells of several hydrozoans. Roosen-Runge (1977) found dense material which he termed nuage in spermatogonia of *Phialidium gregarium*. Kessel (1968a) found densely packed granular aggregates in oocytes of a hydrozoan jellyfish, and Boelsterli (1977) found perinuclear dense finely granulated spherical structures in oocytes of *Podocoryne carnea*. Noda and Kanai (1977) found what they termed 'germinal dense bodies' in the oogonia of *Pelmatohydra robusta* and in lesser quantities in interstitial cells and early nematocytes. More recently, however, the same authors found these bodies in other cell types, including epithelio-muscular, digestive and gland cells (Noda and Kanai, 1980). The possible significance of these bodies in relation to the differentiation of the germ cells is discussed by Nieuwkoop and Satasurya (1981).

Eddy (1975) illustrates how nuage material from different animal groups varies widely in appearance and may alter during germ cell development. The possible nuage material in *A. fragacea* appears more complex in structure than that described for other coelenterates and most other groups. In very small oocytes (less than 12 μm diameter) the nuage is rather regular and consistent in form, but in larger oocytes it tends to be more extensive, more variable in appearance, and often associated with other organelles. However, whether this material plays any part in the determination of the germ cells in *A. fragacea* remains to be demonstrated. Nuage material from later stage oocytes will be discussed in Chapter 6, Section G).

In *Epiactis prolifera*, Dunn (1975) suggested that primary oogonia range in size from 6.0 to 8.5 μm in diameter while germ cells in the endoderm up to 30-35 μm are secondary oogonia. The germ cells cease dividing and become oocytes once they are within the mesogloea. Jennison (1979) describes a similar situation in *Anthopleura elegantissima*. In the present study, however, no evidence of mitotic activity was seen in any of the larger germ cells. In the previous chapter it was shown that endodermal germ cells as small as 10 μm in diameter might contain synaptinemal complexes, suggesting the onset of meiosis. In *A. fragacea* female germ cells there appears to be a process of nuclear maturation in terms of increasing nuclear density and nucleolar development throughout the endodermal phase, which does not appear to be interrupted by mitosis. The larger endodermal germ cells are usually found singly,

scattered throughout the gonad, rather than in groups or nests as might be expected if they were mitotically active. Most of the germ cells accumulate glycogen and some begin synthesis of yolk granules while within the endoderm. It is therefore suggested that, in *A. fragacea*, the germ cells begin meiosis and become oocytes at an early stage while still within the endoderm, before entry into the mesogloea. If mitosis occurs, it is restricted to small cells before the onset of vegetative growth and the accumulation of reserve materials. Schmidt and Schäfer (1980) also indicate that anthozoan female germ cells may become oocytes while within the endoderm and may begin vitellogenesis before entering the mesogloea. However, mitosis has has been observed in the early germ cells of *Cereus pedunculatus* (see Chapter 10).

The migration of the early female germ cells into the mesogloea was described and clearly illustrated with the light microscope by Spaulding (1974) for the sea anemone *Peachia quinquecapitata*, and has since been mentioned by several authors, including Dunn (1975), Jennison (1979) and Szmant-Froelich *et al* (1980). In *A. fragacea* the oocytes appear to move into the mesogloea by a process resembling amoeboid movement, although the endodermal cell bases may also play a part. It is not known whether the oocyte softens the mesogloea around it enzymatically to facilitate entry, but the arrangement of the collagen fibrils immediately in front of the advancing oocyte does not seem to be disturbed or compressed during entry. In *Epiactis*, Dunn (1975) suggested that female germ cells could move

from the endoderm into the mesogloea either by amoeboid movement or at cell division. She suggested that during these divisions one daughter cell remained in the endoderm while the other was thrust into the mesogloea, and she showed an hourglass-shaped cell apparently undergoing this process. No evidence of this means of entry was found in *A. fragacea*. Hourglass-shaped cells are commonly observed at the endoderm/mesogloea border, but they do not appear to be undergoing mitosis. Such cells contain a single nucleus surrounded by an intact nuclear envelope. The constriction does not always divide the cell into two equal parts, but can occur at any position. The cell shown entering the mesogloea in *Peachia* by Spaulding (1974) is also constricted in this way, but has an intact nucleus and shows not signs of mitosis. In *A. fragacea* the constriction appears to be produced where the oocyte passes through the endodermal muscle processes, and might argue that these processes pose more of an obstacle to entry than does the mesogloea itself. It is possible that the constriction of the oocyte may be exaggerated by the contraction of the gonad and mesentery which occurs on fixation, which might cause the muscle processes to pinch the oocyte more severely.

Schmidt and Schäfer (1980) state that the oocyte nucleus is always situated in the last part of the oocyte to enter the mesogloea. In *A. fragacea*, however, this is not usually the case. In oocytes which enter the mesogloea while still small, up to perhaps 15 μm in diameter, the nucleus is often located anteriorly and enters the mesogloea

very early. In larger oocytes the nucleus usually occupies a roughly central position. Only rarely does the nucleus enter very late. At a later stage, after the oocytes have completed entry, the nucleus then migrates to take up a peripheral location close to the trophonema (see below). This movement of the nucleus to one side was also noted for *Epiactis* by Dunn.

Schmidt and Schäfer (1980) describe a vitelline membrane of fine granular material surrounding the surface of anthozoan oocytes, apparently both while within the endoderm and within the mesogloea. For the coral *Astrangia danae*, Szmant-Froelich *et al* (1980) describe a vitelline membrane found only in large oocytes, consisting of a layer of cortical vesicles just beneath the oocyte membrane. The oocytes of *A. fragacea* have no obvious extracellular coat while within the endoderm. As they enter the mesogloea they become covered by a thin layer of very finely granular basal lamina material. From micrographs in which the oocyte is caught in the process of entering the mesogloea, it can be seen that this layer is identical to and continuous with the basal lamina between the epithelial cell bases and the mesogloea, as described for *Anthopleura elegantissima* planulae by Chia and Koss (1979). It appears that as the oocytes enter the mesogloea, they do not break through the basal lamina but rather push it into the mesogloea ahead of them. Once within the mesogloea, the oocyte must presumably produce more basal lamina as it grows. Whether this layer of basal lamina material should be termed a vitelline membrane seems doubtful. This layer is not a structure

specific to oocytes; all cells in contact with the mesogloea, with the exception of granular amoebocytes have such a layer. Groups of developing male germ cells in the mesogloea are surrounded by a similar layer (see Chapter 11). Spawned eggs and early embryos of *A. fragacea* are not surrounded by an extracellular layer of any kind, and neither are the spawned eggs of *Bunodosoma cavernata* or *Tealia crassicornis* (Schroeder, 1982). It is therefore suggested that the term vitelline membrane is inappropriate for the extracellular material around mesogloeal oocytes.

For some *A. fragacea* oocytes, entry into the mesogloea appears to be facilitated by an outgrowth of the mesogloea around them. Mesogloeal outgrowth around oocytes has previously been reported by Dunn (1975) and Schäfer and Schmidt (1980). However, how this outgrowth is brought about is unclear. Singer (1974) investigated collagen synthesis in the sea anemone *Aiptasia diaphana* using electron microscopy and autoradiography and concluded that mesogloeal collagen is synthesized by the epithelial cells. Perhaps the oocyte can stimulate the endodermal cells around it to produce mesogloea. Some small oocytes in *A. fragacea*, while within the endoderm, contact the mesogloea by a slender cytoplasmic process (see Chapter 4). These processes might serve to direct mesogloeal outgrowth as well as movement of the oocyte into the mesogloea. Alternatively, the same forces acting to drive the oocyte into the mesogloea may result in the mesogloea being pulled out around the oocyte.

Once within the mesogloea, the oocyte loses contact with the endodermal epithelial cells over most of its surface. At one point, however, contact with the endoderm is retained and a specialized structure called the trophonema develops which is described in Chapter 7. From the present study, it is possible to speculate and suggest a possible mechanism for the formation of the trophonema in *A. fragacea*. When oocytes have nearly completed entry into the mesogloea, there appears to be a pause in the entry process. During this period, the small proportion of the oocyte remaining in the endoderm enters into a close relationship with the endodermal cell bases immediately around it. This relationship involves close membrane contact, including intercellular junction formation, between oocyte and endoderm, and interdigitation between endodermal cell bases and oocyte cytospines. As the last portion of the oocyte finally enters the mesogloea, some of the endodermal cell bases move or are dragged with it. Thus the gap in the mesogloea/endoderm border through which the oocyte passed never fully closes, and the oocyte retains contact with the endoderm. The endodermal cells involved then begin specialization to become trophonemal cells.

The proposed scheme is broadly consistent with the findings of Schmidt and Schäfer (1980), although some details of the process in *A. fragacea* differ from their description. In particular, Schmidt and Schäfer state that in the early stages the trophonema consists of cells which have no prominent nucleus, have a fine granular cytoplasm and special electron dense bodies. In *A. fragacea* the

trophonema appears to originate from ordinary gonad epithelial cells, indistinguishable from those around them. They may later become specialized, but always retain a normal nucleus (see Chapter 7). Widersten (1965) reports a sequence of events during scyphozoan oogenesis which bears a striking resemblance to the proposed mechanism of trophonema formation described here. However, he refers to the epithelial cells involved as nurse cells, a term which is usually applied to accessory cells derived from germ cells, which, at least in *A. fragacea*, the trophonemal cells do not appear to be.

During the present study, trophonema initiation was not observed for oocytes which enter the mesogloea at a small size (less than 15 μm) and how the trophonema is formed in these cases is not clear. Nonetheless, Campbell's (1974b) suggestion that anthozoan oocytes move into the septal mesogloea at an early stage and appear to mature quite independently with no involvement of somatic cells may now need to be reconsidered.

Chapter 6

THE STRUCTURE OF THE VITELLOGENIC OOCYTE

INTRODUCTION

Vitellogenesis may be defined broadly as the accumulation within the oocyte of reserve materials for use after fertillization (Anderson, 1974). In many invertebrate species vitellogenesis may be the dominant metabolic activity of the oocyte for much of its period of existence within the gonad. In spite of its importance, there has been no coherent study of vitellogenesis in any species of sea anemone (Beams and Kessel, 1983). However, organelles and inclusions from a large number of anthozoan oocytes have been examined by Schmidt and Schafer (1980; Schäfer and Schmidt, 1980; Schäfer, 1983).

Although a small proportion of *A. fragacea* oocytes may begin vitellogenesis while within the endoderm, in most cases major vitellogenesis only takes place after entry into the mesogloea. The process is complex, lasting several months and involving a wide range of organelles and inclusions. Rather than attempt an overall description of the structure of the entire oocyte at different stages during vitellogenesis, individual structures will be considered separately and their development followed throughout this period. The chapter has accordingly been divided into sections, and the relevant literature has been discussed at the end of each section.

The sections within this chapter are:-

Section A - The Nucleus

" B - Endoplasmic Reticulum and Annulate Lamellae

" C - Mitochondria and the Mitochondrial Cloud

" D - Fibrillar Granules

" E - Compound Yolk Granules

Section F - Glycogen and Lipid Droplets

" G - Nuage Material

" H - The Oocyte Surface

" I - Amoebocytes

Because of the large quantity of material included in this chapter, a brief summary of the major findings is given at the end.

Chapter 6, Section A: The Nucleus

RESULTS

During vitellogenesis, the diameter of the oocyte nucleus increases from perhaps 10 μm on entry into the mesogloea, to 40-45 μm in the fully grown oocyte. However, during this period the oocyte diameter increases from perhaps 20 μm to some 150 μm. Thus the nucleus to cytoplasm volume ratio decreases during oocyte growth. The nucleus also changes its appearance during vitellogenesis, as will be described below. Initially the nucleus is centrally located within the oocyte but later it becomes displaced towards the side of the oocyte adjacent to the trophonema. The description of nuclear development has been divided into three sections, but the process is continuous.

1. The Onset of Vitellogenesis

At the time of entry into the mesogloea and the onset of major vitellogenesis, most oocytes are between 12 and 25 μm in diameter. Their nuclei appear dense, and irregular areas of more dense heterochromatin material occur (Fig. 6.1). A single nucleolus can be distinguished, but it is usually associated with the heterochromatin areas. Small dense granules are found, especially in the vicinity of the nucleolus and heterochromatin. The outline of the nucleus is usually smooth or gently undulating, and the nuclear envelope contains occasional pores. As yolk material begins to accumulate in the cytoplasm, the nucleoplasm becomes more dense and the heterochromatin becomes less obvious (Fig. 6.2). The nucleolus increases in size and becomes more discrete and clearly defined. New structures, termed nuclear fibrillar bodies, begin to appear. These consist of aggregations of fibrils, usually

surrounding a core of finely granular material (Fig. 6.3). They may be 1-2 μm in diameter, and up to five such bodies have been seen in a single section through a nucleus at this stage. The fibrils themselves have a beaded appearance, and appear to be composed of particles some 30-40 nm in diameter. The fibrils are usually orientated in a roughly parallel manner. These bodies are usually found near the nucleolus or close to the nuclear envelope.

2. Early Vitellogenesis

During this phase, when the oocyte is between 30 and 60 μm in diameter, the increase in density of the nucleoplasm noticed at the onset of vitellogenesis is reversed. The nucleoplasm becomes less dense, and takes on a finely floccu-lent appearance (Fig. 6.4). Small, irregular areas of more dense, finely granular material may be found scattered randomly through the nucleoplasm, and these may be closely associated with 50-60 nm dense granules (Fig. 6.5). Similar granules may also be found adjacent to the surface of the nucleolus (Fig. 6.6), or in clefts in its surface (Fig. 6.7). Nuclear fibrillar bodies are very commonly found close to the nucleolus, and material resembling their fibrrllar component is often seen projecting from the surface of the nucleolus, or occasionally within surface clefts (Figs. 6.6, 6.7). A very common arrangement is for the nucleolus to be situated near the periphery of the nucleus, close to the nuclear envelope, and for nuclear fibrillar bodies to lie in the space between the two (Fig. 6.8). Finely granular material resembling the core component of the fibrillar bodies may be seen around and in contact with the surface of the nucleolus. Thus there appears to be a very close association between the nucleolus

and the nuclear fibrillar bodies at this stage, and it seems possible that the latter are formed from material originating in the nucleolus.

Where nucleoli and nuclear fibrillar bodies are found close to the nuclear envelope, areas of nuage material are very often found immediately outside it (Fig. 6.8). It seems possible that some interaction between these structures occurs across the nuclear envelope, a possibility which is considered further in Section G of this chapter.

The outline of the nuclear envelope may become less regular, and the number of nuclear pores greatly increases (Fig. 6.4).

3. Mid and Late Vitellogenesis

The nucleus attains its final form during mid-vitellogenesis, at an oocyte diameter of 60-80 μm. It then remains unchanged in appearance, although increasing in size, until the oocyte is fully grown.

The nucleolus may reach a diameter of 10 μm, and is now spherical and homogeneous (Fig. 6.9). At this stage no regions of different composition can be distinguished, and it is no longer found associated with any other structurns. The nucleolus appears to consist of closely packed small granules, each some 8 nm in diameter, embedded in a very finely granular matrix.

The outline of the nucleus is generally smooth, but in localized regions it may show deep invaginations (Fig. 6.10) or extensions (Fig. 6.11). The nucleoplasm is of very low density, and appears to consist of an irregular, finely fibrillar meshwork, interspersed with areas of more dense granular material (Fig. 6.12). In some micrographs, very

small, discrete dense particles, often less than 5 nm in diameter, are also seen. Since these are not visible in all sections, they may represent a fine precipitate formed during processing.

Nuclear fibrillar bodies are commonly found in large oocytes, nearly always close to the nuclear envelope. They are similar to those found in smaller oocytes, except that the fibrils may be more closely packed, and a granular core is not always apparent (Fig. 6.13). Sometimes they are found clustered in groups consisting of 2-4 bundles of fibrils, sometimes with granular cores (Figs. 6.14, 6.15). This clustering was not observed in smaller oocytes.

No signs of the resumption of meiosis were observed in any oocyte within a gonad. Observation of gonads obtained by biopsy from spawning females suggests that meiosis is only resumed at the time of oocyte release from the gonad (see Chapter 13).

DISCUSSION

It has not proved possible to relate nuclear morphology to all the various stages of meiosis passed through in the course of oogenesis. Synaptinemal complexes were found in very small oocytes within the endoderm (up to c. 15 um diameter) but not in later stages (see Chapters 4 and 5). This finding suggests that the late zygotene/ pachytene stages of meiotic prophase are passed prior to entry into the mesogloea and the onset of vitellogenesis. During the onset of vitellogenesis, irregular patches of chromatin material could be distinguished in the nucleus, but no conclusions as to chromosome behaviour or morphology could be drawn. At no stage could lampbrush chromosomes be observed. However, there

are few reports of lampbrush chromosomes occurring in the oocytes of marine invertebrates, although they were described for sea urchin oocytes by Millonig *et al* (1969). There is no evidence to suggest that oogenesis in *A. fragacea* does not follow the common procedure of carrying out vitellogenesis during a long arrested diplotene phase of meiotic prophase, as is thought to occur in a very wide range of animals (Browder, 1980). At the termination of oogenesis, no indications of the resumption of meiosis were observed. No instances of germinal vesicle breakdown were observed in any oocytes while within the gonads, and it is thought that this may occur only immediately before spawning. This is in line with the findings of Jennison (1979) who was unable to find any evidence of meiosis within the gonad of the anemone *Anthopleura elegantissima*, and with those of Szmant-Froehlich *et al* (1980) who concluded that meiosis is resumed just prior to spawning in the coral *Astrangia danae*. Rinkevich and Loya (1979), however, observed germinal vesicle breakdown before spawning in *Stylophora pistillata*.

The appearance of the nucleus in *A. fragacea* is broadly similar to that of many other marine invertebrate species. Scattered, irregular, dense chromatin areas have been described for many early oocytes, including the annelids *Branchiobdella pentodonta* (Bondi and Facchini, 1972), *Phragmatopoma lapidosa* (Eckelbarger, 1979), *Streblospio benedicti* (Eckelbarger, 1980) and the molluscs *Ilyanassa obsoleta* (Taylor and Anderson, 1969) and *Mopalia mucosa* (Anderson, 1969). These authors, however, do not report the quite striking increase in nuclear density seen in *A. fragacea* during previtellogenic oocyte growth.

Throughout oogenesis, the oocyte nucleolus in *A. fragacea* appears homogeneous, although it may interact with other structures. The anemones *Epiactis prolifera* (Dunn, 1975) and *Anthopleura elegantissima* (Jennison, 1979) also have single, spherical, homogeneous nucleoli. Oocyte nucleolar structure is often more complex, however, A degree of vesicularization of the nucleolus is common in many invertebrate groups (Norrevang, 1968) and some partitioning of the nucleolus into morphologically distinct regions is also common (Anderson, 1974). A large vacuole develops in the centre of the nucleolus during oogenesis in the hydrozoan *Sertularella polyzonias* (Honegger, 1980) and *Pennaria tiarella* (Cowden, 1964). The nucleoli of the unidentified trachyline medusa studied by Kessel (1968a) had a tripartite structure, and Widersten could distinguish cortical and medullary regions in the nucleoli of several scyphozoan oocytes (Widersten, 1965). *Cereus* oocyte nucleoli may also be vacuolated (see Chapter 10).

In *A. fragacea*, it seems possible that the nuclear fibrillar bodies are formed with the involvement of the nucleolus, and then migrate to take up a position close to the nuclear envelope. No evidence of the bulk passage of material through the nuclear pores was obtained in the present study. Such bulk transport has been frequently reported in the literature, however, and the material passing out through the nuclear pores is often thought to be of nucleolar origin (Anderson, 1974). An example which shows some parallels with *A. fragacea* is provided by ophiuroid echinoderm *Ophioderma panamensis* (Kessel, 1968b). In this species, the oocyte nucleus contains several nucleoli, and the outer region of each nucleolus appears less closely packed than the centre.

A branching system of strands or threads emanates from the periphery of many nucleoli. It is suggested that the nucleoli undergo fragmentation by the spinning out of threads which later break down into smaller pieces. These are commonly found in close proximity to the nuclear envelope, and especially near the nuclear pores. Similar material is also found in the cytoplasm immediately adjacent to the nuclear envelope, but fragments have not been seen within the pores themselves.

Structures very similar, at least in appearance, to the nuclear fibrillar bodies of *A. fragacea*, have been described from oocytes of the slug *Agriolimax reticulatus* (Hill and Bowen, 1976). They are often found embedded in the nucleolus, but during the later stages of oogenesis they are found at the periphery of the nucleolus and free within the nucleus. It is therefore thought that they are released from the nucleolus, but their function is uncertain.

Fibrillar nuclear bodies have not been reported from other coelenterate species. They occur in *Cereus pedunculatus* (see Chapter 10), and very similar structures are shown lying next to the nucleolus of a *Cerianthus membranaceus* oocyte in a micrograph by Schäfer and Schmidt (1980), but are not mentioned in the text. If, as seems possible, in *A. fragacea* they interact with nuage material lying outside the nuclear envelope, it would appear that this interaction occurs by the passage of very small particles or soluble material through the nuclear pore. Beams and Kessel (1963) describe how material from the nucleolus may break down into small particles, pass through the nuclear pores and then reaggregate into small masses in the cytoplasm of the crayfish oocyte. Interestingly, the

nucleoli in this species are located close to the nuclear envelope, and the shedding of material is more common from the side of the nucleolus closest to the envelope, as would appear to be the case in *A. fragacea*. In *A. fragacea*, of course, there is no reason to suppose that the flow of material, if any, must occur from nucleolus to cytoplasm. Movement in the reverse direction is perfectly possible on the evidence so far obtained. The nuage material is considered in more detail in Chapter 6, Section G.

Many authors have reported an increase in the density of pore complexes in the nuclear envelope at the onset of vitellogenesis. Anderson (1974), however, points out that quantitative experiments are needed to investigate the relationship (if any) between the porosity of the envelope and the metabolic involvement of the oocyte's synthetic machinery. Similarly, Anderson and Huebner (1968) discuss the possible relationship between the folding or plication of the envelope and the rate of synthetic activity within the oocyte. Such foldings must increase the area of the nuclear envelope, which, coupled with the high density of pore complexes, may facilitate nucleo-cytoplasmic interchange. Such exchange may be particularly important in oocytes like those of *A. fragacea* which appear to carry out a great deal of intraoocytic synthesis of yolk protein (see following sections), presumably under the direction of the oocyte genome.

The pore complexes in *A. fragacea* are conventional in appearance. They appear to be sealed by a diaphragm, but, as suggested by Barnes and Davis (1955) and Kessel (1968c) this is probably merely a sectioning effect. Throughout oogenesis,

the nuclear membrane appears to be of constant width and shows no signs of blebbing or vesicularization as occurs in many oocytes (Kessel, 1968c; see also following section, 6.B).

Chapter 6, Section B: Endoplasmic Reticulum and Annulate Lamellae.

RESULTS

1. The Endoplasmic Reticulum (ER)

The very smallest oocytes contain little or no discernible ER (see Chapter 4). By the time the oocytes reach 10-12 μm diameter, some ER is visible, usually in the form of short, irregular lengths randomly distributed through the cytoplasm (Fig. 6.16). These lengths are usually heavily studded with ribosomes. During subsequent previtellogenic growth, oocytes vary greatly in the amount and the distribution of the ER they contain. Many oocytes accumulate small areas of stacked ER cisternae widely scattered through the cytoplasm, sometimes in addition to single lengths (Fig. 6.17), while others contain ER arranged around the nucleus. Rarely, previtellogenic oocytes are found containing highly ordered ER stacks in the peripheral cytoplasm (Fig. 6.18). In general, it can be said that there is a gradual, if variable, increase in both the quantity and degree of organization of the ER during the previtellogenic period.

After entry into the mesogloea and the onset of vitellogenesis, there is a marked increase in the quantity of ER within the oocyte. Very early in vitellogenesis, some oocytes are found in which the cytoplasm contains numerous randomly arranged lengths of ER (Fig. 6.19), but in most cases, and always in later stages, the ER is arranged in roughly parallel arrays. These can occur both in the peripheral cytoplasm and folded around the nucleus.

The peripheral stacks of ER may be 5 μm long in section, and, given their often curved or undulating appearance, may be more extensive in 3 dimensions. Up to 15 cisternae commonly lie together to form a stack (Fig. 6.20). The cisternae may run without apparent interruption for long intervals, or may appear to be broken up into shorter lengths (Fig. 6.21). The cisternae contain a material of moderate electron density, and are generally narrow and without major dilations. In localized areas, the cisternae appear collapsed, and show only a very narrow lumen. The peripheral ER stacks often appear to be associated with other cytplasmic inclusions, most notably Golgi complexes (Fig. 6.22), but also lipid droplets, an association which will be considered in Chapter 6, Section F .

During mid and late vitellogenesis, the greater proportion of the ER is found around the oocyte nucleus. The ER forms a band which is usually immediately adjacent to the nuclear envelope, although they may be separated for short lengths. This perinuclear band is not of equal thickness all around the nucleus. Some parts have no covering at all; most have a moderate covering, and in some areas the ER forms large masses. Most of the nucleus is covered by a band some 10-15 cisternae thick, which usually follows the contours of the nuclear envelope (Fig. 6.23). The cisternae are generally parallel and narrow, and may be continuous for long distances, or may be interrupted by gaps, usually about 300 nm wide. These gaps may represent fenestrations in the sheet-like cisternae (Fig. 6.13). Sometimes other organelles or inclusions are found in amongst the ER band, or between it and the nuclear envelope (Fig. 6.24).

Over limited regions of the nuclear surface, the band of ER may be much more extensive. Sometimes the cisternae in these regions remain flat or gently undulating (Fig. 6.25), but more commonly the ER is pleated or domed. These foldings are sometimes accompanied by similar irregularities in the underlying nuclear envelope (Fig. 6.26), but this is not always the case (Fig. 6.27). These ER masses may be upwards of 10 μm thick, and consist of over 50 stacked cisternae. They may occupy a considerable proportion of the oocyte volume, as in Fig. 6.28, where the perinuclear band extends almost to the surface of the cell. The cisternae in these large masses are also interrupted by fenestrations, and small areas of some cisternae appear collapsed (Fig. 6.29). Ribosomes are less commonly found attached to these collapsed regions. Occasionally myelinic figures are found associated with the cisternae (Fig. 6.29). Usually these very large areas of ER are uncontaminated by other cell components, but occasionally mitochondria, lipid droplets or yolk granules are found surrounded. Rarely, the ER impinges on other perinuclear structures such as the mitochondrial cloud (Fig. 6.27) and in this case some intermingling of the organelles may occur. Extensions from the perinuclear ER sometimes project out into the cytoplasm. Fig. 6.31 shows part of one such extension, which could be followed through the cytoplasm for some 15 μm. The composition of the ooplasm on its two sides is markedly different, suggesting that it may represent an effective and relatively stable barrier, at least to large inclusions.

Occasionally, regions of more dilated ER cisternae were encountered, associated with both peripheral and perinuclear ER areas (Fig. 6.30). These appear as ribosome-studded sacs,

perhaps 300 nm in diameter. Some sacs appear more flattened or elongate, but many appear spherical. They are filled with a flocculent, finely granular material of only moderate electron density. Areas of this form of ER seem more likely to be interspersed with other inclusions, often glycogen, than other areas.

Small whorls of apparently tightly packed ER cisternae are sometimes found around the periphery of perinuclear ER masses (Fig. 6.32). The cisternae within these whorls are narrower than usual, although some contain material of moderate electron density. It is not possible to discern ribosomes between the tightly packed cisternae. The whorl shown in Fig. 6.32 appears to have an expanded region to one side, with more normally spaced cisternae which are liberally covered with ribosomes.

When the oocyte has attained its final size and vitellogenesis is complete, the organized ER masses appear to fragment and become less organized, then disperse in the ooplasm. Spawned eggs contain no perinuclear ER, and only a few randomly arranged short lengths occur in the peripheral ooplasm (see Chapter 13).

2. Annulate Lamellae

Annulate lamellae were found in oocytes of all sizes. Their development appears to parallel that of the ER, such that they are plentiful during the later stages of vitellogenesis but less common in previtellogenic or fully grown oocytes. Each annulate lamella closely resembles a portion of nuclear envelope, and contains numerous pore complexes identical to the nuclear pores. Annulate lamellae tend to be straighter than most areas of the nuclear envelope, and the pore complexes are always closely and regularly packed, which is not always

true for the nuclear envelope. The lamellae often occur in parallel stacks, in which case the pore complexes are usually more or less aligned.

In small oocytes, the annulate lamellae usually occur as relatively small stacks of 2-6 lamellae, and tend to occur free within the cytoplasm (Figs. 6.33, 6.34) and not associated with other organelles. The membranes may extend for a short distance beyond the pore complexes, and may be studded with ribosomes, but otherwise they are not related to the ER.

In vitellogenic oocytes, however, the annulate lamellae are always found associated with the ER system. The largest annulate lamellae accumulations were found among the perinuclear bands of ER (Fig. 6.35). In each case the membranes are seen to be continuous between the two organelles. At high magnification (Fig. 6.36), it can be seen that dense material is associated with the pore complexes. The pores themselves often appear to be closed by a diaphragm, but this is probably artefactual. Ribosomes are found between the individual lamellae. Areas of annulate lamellae may include discontinuities or smaller regions in which the organization appears to be lost and which seem to contain only membrane fragments and free ribosomes (Fig. 3.37).

In a small proportion of perinuclear ER masses, the annulate lamellae only occur in the innermost few ER cisternae, and are only found in close apposition to the nuclear envelope (Fig. 6.38). More commonly, however, they are found some distance from the envelope (Fig. 6.39) or even at the periphery of the ER mass. They are also found in peripheral ER masses (Fig. 6.40), although these stacks are usually smaller.

While most annulate lamellae occur in relatively large

stacks, single or double lamellae are occasionally found. In particular they may be found curved around the surface of large individual compound yolk granules (Fig. 6.41). Sometimes these annulate lamellae are connected to ER cisternae, but the ER is rarely associated with the yolk. This association would appear to be specific, since neither ER nor annulate lamellae have been found curved around fibrillar granules or cortical granules. ER is often curved around lipid droplets, but annulate lamellae have never been seen to do so.

DISCUSSION

It is now generally accepted that the ribosome-studded or rough endoplasmic reticulum (RER) is a major site for the synthesis of proteins in a wide variety of cell types. An elegant model for the synthesis of protein by the RER has been proposed by Blobel and co-workers, among others (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975). Briefly, messenger RNA molecules pass from the nucleus to the cytoplasm, where they associate with ribosomal subunits and begin translation. The first part of the polypeptide chain to be assembled contains a hydrophobic 'signal sequence'. When the ribosome contacts an ER membrane, the signal sequence attaches to, and penetrates the membrane, possibly assisted by a specific signal recognition protein. As the polypeptide chain elongates, it passes into the lumen of the ER. The signal sequence is then cleaved from the chain, which when completed is released into the lumen. Further processing and transport of the protein molecule may then take place in the lumen. The finding that very large quantities of RER accumulate in *A. fragacea* oocytes during vitellogenesis, but regress once vitellogenesis

is complete, therefore argues strongly for its involvement in the synthesis of yolk protein, and suggests that this synthesis occurs on a large scale. The synthesis of yolk protein within the oocyte has been termed autosynthesis (Schechtman, 1955) in contrast with heterosynthesis, in which yolk protein is produced at an extra-oocytic site and subsequently taken up by the oocyte. Further details of autosynthetic yolk production in *A. fragacea* will be given in later sections in this chapter. Autosynthesis has been reported from a large number of invertebrate species (Anderson, 1974), and these oocytes generally contain a well developed RER. In contrast, oocytes in which heterosynthesis predominates tend to contain relatively little RER, although the smooth ER may be extensive (e.g Dumont and Anderson, 1967). An interesting exception is provided by the proturan *Acerentomon gallicum* (Bilinski, 1977). Yolk production is thought to be entirely autosynthetic in this species, but the small amount of RER found in early oocytes disappears early during vitellogenesis. It is suggested that the major contribution to yolk protein synthesis is made by free ribosomes in this species.

The majority of the RER in the *A. fragacea* oocytes is organized as large sheets layered concentrically around the nucleus. In many species, the RER is reported as being continuous with the nuclear envelope, but no such connections were observed. Similarly, no connections were obvious between adjacent ER cisternae. The numerous fenestrations observed in the cisternae may provide a means of cytoplasmic transport around the ER system, which may be important to allow access of protein precursors, including mRNA and amino acids to the surface of the ER membranes, and to allow communication across

the ER masses. Peripheral ER bands do seem to pose an effective barrier to the movement of large ooplasmic inclusions, since the composition of the ooplasm may appear very different on the two sides of such bands.

The perinuclear masses of RER found in the *A. fragacea* oocytes seem larger and more highly ordered than those found in other invertebrate oocytes. Oocytes of the ophiuroid *Ophioderma panamensis* contain extensive bands of RER just beneath the cell surface (Kessel, 1968b) but these do not appear to be as massive as found here. Among the coelenterates, Schafer (1983) reports that the RER is developed to a high degree in hexacorallian oocytes, but does not illustrate its extent. The oocytes of the hydrozoans *Hydra* (Honegger, 1983), *Tubularia* (Boelsterli, 1975) and *Podocoryne* (Boelsterli, 1977) do not accumulate proteid yolk, and contain little RER.

A similar situation appears to exist in ooyctes of the anthomedusan *Spirocodon saltatrix* (Kawaguti and Ogasawara, 1967). However, oocytes of the unidentified trachyline medusa investigated by Kessel (1968a) accumulate yolk bodies and contain an extensive RER. In this species, the RER displays an unusual association with another membrane system. It appears that this second system consists of a series of deep invaginations of the oolemma. In some areas, the ER is alternately positioned between these invaginations, so the two membrane systems are arranged in a stacked, parallel array with a tight alignment apparent. The second membrane system also becomes involved with the forming yolk bodies for a period in their development. No comparable membrane system was observed in *A. fragacea* oocytes.

The reduction in quantity and organization of the RER seen at the end of vitellogenesis in *A. fragacea* has also been observed in other species, including the annelids *Branchiobdella pentodonta* (Bondi and Facchini, 1972) and *Phragmatopoma lapidosa* (Eckelbarger, 1979). Large masses of ER might interfere with communication within the egg or possibly hinder cleavage, and since levels of protein synthesis are generally low in mature eggs (Epel, 1967, 1975) such a reduction may be of value. However, in the mollusc *Mopalia*, Anderson (1969) reports that the quantity of ER does not fall as the egg matures. He suggests that it exists in a quiescent state after the completion of vitellogenesis, but may serve as a reservoir of protein synthetic machinery for use later in development.

Annulate lamellae

The term annulate lamellae was first used by Swift (1956) although the structures had probably been observed some years earlier in *Arbacia* eggs by McCulloch (1952). Since then they have been reported from a number of cell types in a wide range of animal species. They appear to be most commonly found in developing male and female germ cells, certain embryonic cells and in tumour cells. Less commonly, they may occur in adult somatic cell types (Kessel, 1968c). In spite of their wide-spread occurrence, there is no general agreement as to their mode of formation, or their function. Several suggestions have been made. The close similarity in appearance between the annulate lamellae and the nuclear envelope has prompted many authors to seek the origin of the annulate lamellae there. Rebhun (1961) suggested that they arise by a process of delamination of the entire nuclear envelope. Barer, Joseph and Meek (1960) suggested that their formation involved

repeated folding of the nuclear envelope. Harrison (1966) considered that they might be formed on the nuclear membrane, perhaps using it as a template. In his major review, Kessel (1968c) proposes a scheme largely based on a study of oocytes of the amphibian *Necturus* and the echinoderm *Thyone*. Briefly, it proposes that extensive blebbing of the outer membrane of the nuclear envelope results in the formation of long chains of vesicles in linear array in the cytoplasm immediately outside the nucleus. These vesicles then migrate to the peripheral cytoplasm, still in linear array, where they fuse to form cisternae. Pore complexes then arise in the cisternae, possibly at the points where vesicle fusion took place. Kessel (1968c) makes several speculations as to the possible function of the annulate lamellae, including a role in the transport of material, and possibly information, from the nucleus into the cytoplasm. Wischnitzer (1970) attaches greater importance to the close relationship between the annulate lamellae and the ER. He gives support to the earlier suggestion of Hruban *et al* (1965) that "annulate lamellae may represent an intermediate stage in the formation of the endoplasmic reticulum." He suggests that they may be transient structures, and concludes that they may serve as an accessory means of increasing the ergastoplasmic pool in times of high metabolic demand, and proposes a mechanism by which ER cisternae grow out from the distended ends of the annulate lamellae. In a more recent review, Maul (1977) simply reports that there are no known functions for annulate lamellae. He then lists a number of points which may have to be considered in the formulation of hypotheses concerning their function, but does not indulge in any speculation.

In *A. fragacea*, annulate lamellae are occasionally found in isolation in previtellogenic oocytes, but in later stages they were always found in continuity with the RER. Their close association with the ER in anthozoan oocytes was also noted by Schäfer (1983). They were sometimes found closely apposed to the nuclear envelope, but even in this position, where they might be expected to have recently formed, they were still continuous with the ER. No evidence of blebbing of the nuclear envelope was obtained. Little can be proposed as to the formation or function of the annulate lamellae in *A. fragacea* oocytes. They could perhaps most simply be regarded as localized areas of the RER which are specialized and contain pore complexes. The membrane regions between the pore complexes are studded with ribosomes and are identical to RER membranes. It has been suggested that, after mitosis, the nuclear envelope and its pore complexes are formed from the ER (Barer *et al*, 1959). The nuclear envelope is widely reported to have many similarities with the ER. The two are often continuous, both are studded with ribosomes and the nuclear envelope may be involved in protein synthesis (Hand and Oliver, 1977). The present study confirms the close relationship between the three organelles, but does not allow conclusions about their relative origins to be drawn.

RESULTS

The very smallest oocytes have rather few organelles, and the few mitochondria they contain tend to be dispersed through the cytoplasm (see Chapter 4). As the oocytes enlarge, more mitochondria become apparent. They tend to be small and rounded, measuring about 0.4 x 0.6 μm in section. They have a homogeneous dense matrix tranversed by numerous narrow cristae. In a proportion of mitochondria, especially in the larger profiles, the cristae may show an unusual lattice-like arrangement. Many of these cristae are triangular in cross-section, and so are thought to be prismatic in three dimensions (Fig. 6.42). Some mitochondria may also contain a central region of dense matrix material which is not traversed by cristae (Fig. 6.43). These forms of mitochondria are less common in oocytes larger than about 15 μm in diameter.

As the oocytes grow within the endoderm and the numbers of mitochondria they contain increase, the mitochondria tend to become arranged in groups rather than scattered through the cytoplasm (Figs 6.44, 6.45, 6.46). These groups are usually found close to the nucleus but occasionally occur more peripherally in the cytoplasm (Fig. 6.47). The groups do not always contain all the mitochondria of the oocyte; apparently isolated mitochondria are often found. Nuage material is very often found associated with the groups of mitochondria (Figs. 6.45-6.47). Actual contact between nuage and mitochondria has not been observed, however, and occasionally nuage is found not apparently associated with any mitochondria.

Towards the end of the pre-vitellogenic phase, usually soon after entry into the mesogloea and at an oocyte diameter

of 20-30 μm, a group of mitochondria lying close to the nucleus begins to enlarge. This presumably occurs by replication of the mitochondria within the group, although other groups and single mitochondria may become incorporated into the group as it expands. The perinuclear group comes to resemble the mitochondrial clouds which have been described from other species (see Discussion). During the early stages of cloud formation, nuage material and Golgi complexes are often found within it (Figs. 6.48, 6.49).

As major vitellogenesis gets under way, the cloud enlarges further, and nuage material is only rarely found within it (Fig. 6.50). At this stage, usually between 30 and 50 μm in diameter, the oocytes develop an extensive band of endoplasmic reticulum, which, as described in the previous section, may encircle much of the nucleus. Occasionally, the endoplasmic reticulum may extend into the perinuclear region occupied by the mitochondrial cloud, and may disrupt it (Fig. 6.51). More commonly, however, the cloud remains relatively compact, although it may be irregular in outline. The mitochondria are not tightly packed within the cloud, and ribosomes and short lengths of endoplasmic reticulum are found interspersed with them. The cloud reaches its greatest size usually mid-way through vitellogenesis, at an oocyte diameter of perhaps 80 μm, and may itself be 12-15 μm across (Fig. 6.52). While the cloud may contain a high proportion of the mitochondria of the oocyte, small groups and individual mitochondria may still be found elsewhere in the ooplasm.

During the early stages of cloud formation, the great majority of the component mitochondria retain their original appearance. Most appear squat and rounded in section, although

occasional more elongate profiles are seen. In larger oocytes, however, the cloud may contain many highly elongate mitochondria which are often irregular in shape (Fig. 6.53). Mitochondrial profiles, perhaps only 150 nm wide, can be found extending 4-5 μm in section: given their often irregular shape, they may be expected to be considerably longer in three dimensions. Many of the elongate mitochondria are curved or sinuous, and circular or ring-shaped profiles are often seen (Figs. 6.54, 6.55). In many cases the cristae appear sparse or poorly defined. It is difficult to establish the three-dimensional appearance of these larger clouds from random thin sections, but the impression obtained is that of a tangled mass of elongate, irregular mitochondria, rather than an accumulation of squat individuals as found in smaller oocytes.

Later still, the cloud tends to become more irregular in shape. Bands of mitochondria may extend out from the main body of the cloud for a considerable distance into the surrounding ooplasm (Fig. 6.56). Usually at about the time when cortical granules start to appear in the peripheral ooplasm, at an oocyte diameter of perhaps 100 μm, the mitochondrial cloud appears to break up. Groups of mitochondria seem to separate from the main mass and can be found scattered throughout the oocyte. These in turn disperse, and other cytoplasmic inclusions such as yolk granules, lipid droplets, fibrillar granules and cortical granules are found interspersed with the mitochondria (Fig. 6.57). Elongate profiles are rarely found after the onset of cloud dispersal. In the fully grown oocyte, no indications of mitochondrial aggregation remain. They are usually found singly, although they do show a degree of zonation within the oocyte. They are

more common in the peripheral layers of the oocyte, in contrast with their previous perinuclear location.

In fully grown oocytes, most mitochondria are rounded in section and have a dense matrix with numerous cristae which tend to be rather haphazardly arranged (Fig. 6.58). The prismatic cristae found in small oocyte mitochondria have not been observed. In some oocyte sections, however, a proportion of the mitochondria have a rather different appearance. In these cases, the matrix is very much less electron dense, and may include areas which appear virtually clear (Fig. 6.59). The cristae are usually few in number, but are narrow and of fairly constant width. This different appearance of some mitochondria may indicate an alteration in their physiological state, or could merely reflect poor preservation during fixation. However, they may be found in oocytes in whicn all the other cell components appear to be well preserved.

DISCUSSION

Mitochondria with prismatic cristae, while not widespread, have been reported on a number of occasions, both from vertebrates (e.g Blinzinger *et al*, 1965; Morales and Duncan, 1971) and invertebrates (e.g Fain-Maurel, 1968; Murdock *et al*, 1977). Some of these mitochondria also contain an array of rodlets, and Murdock *et al* (1977) have proposed a hypothesis that these rodlets are important in establishing the geometric arrangement of the cristae. The mitochondria with prismatic cristae found in *Actinia fragacea* early oocytes are similar in appearance to those described by Murdock *et al* from crayfish vas deferens sphincter muscle, but do not appear to possess intramitochondrial rodlets. It therefore seems

unlikely that their proposed mechanism is operating in this case. It is interesting that, in both cases, only a proportion of the mitochondria in a given region of tissue exhibit this form; they may be intermingled with mitochondria with a more typical arrangement of cristae.

During the course of oocyte growth in *A. fragacea*, the mitochondria come to form a large mass close to the nucleus, which is similar in appearance to the mitochondrial clouds of other animal species. Mitochondrial clouds have been reported from a number of invertebrate species. Recently, Eckelbarger (1979) describes the occurrence of a mitochondrial cloud in pre-vitellogenic oocytes of the polychaete annelid *Phragmatapoma lapidosa*. Anderson and Huebner (1968) also note the accumulation of mitochondria at the animal pole of oocytes of the polychaete *Diopatra cuprea*. However, the mitochondrial cloud has been most extensively studied in amphibians, and in particular for the anuran *Xenopus laevis*. In spite of the wide phylogenetic separation, parallels can be drawn and comparisons made between the clouds of *A. fragacea* and *Xenopus*.

In small *A. fragacea* oocytes, mitochondria are often found in clusters which may have a perinuclear location, as is the case in young oocytes of many species (Anderson, 1974). One such perinuclear cluster enlarges and eventually forms the mitochondrial cloud. In *Xenopus*, the cloud arises by the coalescence of several smaller mitochondrial aggregates, which initially are dispersed evenly around the germinal vesicle. They come to form a cap-like structure on one side of the germinal vesicle (Billett and Adam, 1976). In *A. fragacea*, it is not clear whether migration of other mitochondrial

groups to join the developing cloud takes place, or whether one perinuclear cluster merely prolliferates much more rapidly than the others. In *Xenopus*, during the early stages, the cloud is located in a large depression in one side of the germinal vesicle, which was not observed in *A. fragacea*. Also, Tourte *et al* (1981) suggest that, in *Xenopus*, there is a precise localization of the cloud with respect to the orientation of the nucleus and the Golgi apparatus during early oogenesis. No favoured position for the cloud on the surface of the nucleus could be discerned during the present study.

The mitochondrial cloud in *A.fragacea* appears to be less regular in shape and less clearly defined than in *Xenopus*. There, the cloud is roughly spherical, can be distinguished with the light microscope within the intact oocyte, and can be removed relatively intact by microdissection (Billett and Adam, 1976). In *A. fragacea*, the cloud, especially late in its development, may be ragged in outline, and bands of yolk granules or endoplasmic reticulum may extend into it.

In *Xenopus* the cloud, in addition to mitochondria, may contain large numbers of small vesicles, 0.1 to 0.2 μm in diameter. The relative number of these vesicles increases as the cloud enlarges (Billett and Adam, 1976). No such vesicles were observed within the cloud in *A. fragacea*, although it may contain ribosomes and short, randomly arranged cisternae of rough endoplasmic reticulum.

Aggregates of mitochondria are found associated with nuage material in *Xenopus* primordial germ cells and oogonia (Al-Mukhtar and Webb, 1971), but this association is not a characteristic feature of the mitochondrial cloud in the

oocyte (Billett and Adam, 1976). An association between mitochondria and nuage occurs in *A. fragacea* but seems less intimate than in *Xenopus*, since direct contact between the two was never observed, and areas of nuage were found apparently not in association with mitochondria. A point of similarity is that the association is more noticeable during the early stages of oogenesis. In *A. fragacea*, nuage may be found among mitochondrial goups in small oocytes and in clouds early in their development, but is not normally seen in large mitochondrial clouds. Billett and Adam (1976) conclude that it is more reasonable to suppose that the nuage material may be some kind of germ cell determinant rather than a substance essential for mitochondrial replication. In *A. fragacea*, nuage material identical to that seen in oocytes is also found in early male germ cells, which do not undergo massive mitochondrial proliferation. This finding might also argue for a wider role for nuage than involvement in replication of mitochondria (see Section G of this chapter).

In *Xenopus*, the cloud is thought to be the site of rapid mitochondrial proliferation, and is an active site of mitochondrial DNA synthesis (Billett, 1979; Tourte *et al*, 1981), although the level of activity of the cloud may vary considerably with the age of the female frog (Callen *et al*, 1980). Billett and Adam (1976), using a combination of conventional transmission electron microscopy, high voltage electron microscopy of thick sections, and scanning electron microscopy of isolated clouds, concluded that the *Xenopus* cloud consisted of tangled mass of very long mitochondria, reminiscent of spaghetti, rather than individual short mitochondria. Although only conventional TEM of thin sections

was employed in the present study, the available evidence points to there being a possibly similar situation in the mitochondrial cloud of *A. fragacea*. Highly elongate mitochondria, and curved or irregular mitochondrial profiles, were frequently encountered in well-developed clouds, but were rarely observed prior to cloud formation or after its dispersal. The upper limit of the lengths of these elongate mitochondria could not be estimated, but, given their usually tortuous shape, is likely to be in excess of the lengths seen in profile in thin sections. Circular or ring-shaped mitochondrial profiles, as found in *A. fragacea*, have not been described from *Xenopus* clouds. They have, however, been observed in previtellogenic oocytes of the mollusc *Ilyanassa obsoleta* (Taylor and Anderson, 1969), and Anderson (1974) indicates that 'donut-shaped' mitochondria may occur during oocyte development in many animals.

The timing of the development of the mitochondrial cloud is rather different in *A. fragacea* and *Xenopus*. In *A. fragacea* the cloud may become apparent at about the time of the onset of vitellogenesis, and may persist as a recognizable, although more diffuse, structure well into vitellogenesis, until at least the time of cortical granule appearance. In *Xenopus*, the cloud forms, grows and disperses all within the pre-vitellogenic phase of oocyte growth. The mechanism of yolk formation is very different in the two species, which may make different metabolic demands upon the oocyte. In *Xenopus*, yolk formation is thought to be heterosynthetic, with yolk protein synthesis largely taking place remote from the oocyte, in the the liver, (Wallace and Dumont, 1968), and there is a well-defined previtellogenic phase. In *A. fragacea*, it is likely that

significant amounts of yolk protein synthesis take place within the oocyte itself. The pre-vitellogenic phase is poorly defined, some oocytes beginning to accumulate yolk granules at a small size, sometimes prior to entry into the mesogloea. Thus the demands on mitochondria during oocyte growth may be very different in the two species.

The finding that fully grown *A. fragacea* oocytes may contain a proportion of mitochondria of unusual appearance may simply be the result of variable fixation and so have no functional significance. However, since other oocyte components appear well preserved, their different appearance may reflect a different physiological state of some mitochondria. It has been suggested that oocyte mitochondria serve two purposes, firstly that of providing for the metabolic needs of the oocyte, and secondly to serve as a store of mitochondria to meet the early requirements of the developing embryo (Billett, 1979). Thus oocytes contain a mixture of mitochondria, possibly differing both in structure and function. The metabolic needs of a fully-grown oocyte may be very different from those of a growing vitellogenic oocyte, especially one engaged in autosynthesis of yolk material such as *A. fragacea*. It may be that when the demands of yolk production decrease, some oocyte mitochondria enter a state of lower activity, which may be reflected morphologically.

In the present study, no evidence was obtained that mitochondria enter the *A. fragacea* oocyte from outside, or are produced from other cytoplasmic structures. Mitochondrial proliferation within the oocyte appears to result in the formation of a large aggregate of possibly elongate mitochondria which may be termed a mitochondrial cloud, a

structure not previously reported from coelenterate oocytes. Interestingly, the mitochondrial cloud of this lower metazoan shows many similarities with the clouds described for oocytes of some amphibian species.

Chapter 6, Section D: Fibrillar Granules

RESULTS

The term 'fibrillar granule' is here being applied to a range of membrane bound bodies whose contents have a fibrillar substructure. Some contain a closely packed mass of parallel fibrils, while in others the fibrillar arrangement is barely discernible. However, all the various forms are thought to be related. The closely-packed form is perhaps the most distinctive, and will be dealt with first.

1. Closely Packed Fibrillar Granules

These are usually slightly elongate bodies, between 1 and 2 μm in length and 0.5 to 1 μm in width. They are bounded by a typical 10 nm trilaminar unit membrane, which is usually ruffled in outline rather than smooth. The granules contain numerous roughly parallel fibrils orientated along the long axis of the granule, and usually extending for its whole length (Fig. 6.60). The fibrils are not always perfectly straight, but are often gently curved. When the granules are cut transversely, it can be seen that each individual fibril is X-shaped in cross section (Fig. 6.61). Each fibril measures some 15 nm across the arms of the X, and at high magnification, there is at least the suggestion that each fibril is composed of smaller granular subunits (Fig. 6.62). The fibrils are arranged in a fairly, but not precisely, regular lattice, with a centre to centre distance of about 25 nm. Especially in larger granules, the plane of the fibrils may change gradually across the granule (Fig. 6.61). The spaces between the fibrils appear to contain a finely granular material.

Quite commonly, the fibrils do not extend fully to the

ends of the granule, which are instead filled with a non-fibrillar granular material of similar electron density to the rest of the granule (Fig. 6.63). Less commonly, the non-fibrillar material also extends around the lateral faces of the granule, and the fibrillar component may only represent a relatively small proportion of the granule (Fig. 6.64). Occasionally, granules of this type may also contain small spherical vesicles, each some 30 nm in diameter (Fig. 6.65) which may also show a linear arrangement.

Often, especially in larger granules, the orientation of the fibrils may change abruptly within the granule. It seems possible that this is the result of previous fusion between two or more granules, and occasionally what may be instances of such fusion are seen (Fig. 6.66).

2. Loosely Packed Fibrillar Granules

In addition to the rather regular, closely packed fibrillar granules described above, a whole spectrum of less regular, more loosely packed fibrillar granules are commonly found. The fibril packing also has a marked influence on the overall electron density of the granule, such that loosely packed granules appear much less dense than closely packed ones. Loosely packed granules may be slightly larger than most closely packed ones. A low power view of an area of ooplasm containing numerous fibrillar granules of varying packing densities is given in Fig. 6.67. In the less extreme cases, the inter-fibrillar spacing is greater than in closely packed granules, and is much more variable within the granule (Figs. 6.68, 6.69) but the fibrils themselves are well defined relatively straight, and are aligned along a common axis. The limiting membrane is usually undulating and often loosely

fitting, such that clear space often exists between the membrane and the granule contents. In other cases, the fibrils may be wavy and less clearly defined (Fig. 6.70), and in extreme cases, the fibrillar nature of the granule contents may be only just discernible (Fig. 6.71). Small dense particles, some 8 nm in diameter, are also found within these loosely packed granules, interspersed with the fibrillar material. The limiting membrane often appears disrupted and discontinuous. Rarely, granules with quite clearly defined fibrils are found which completely lack a limiting membrane (Fig. 6.72). Most loosely packed granules appear to consist of a single bundle of fibrils aligned along a single axis, but occasionally granules containing several bundles are seen, and granules apparently in the process of fusing are also sometimes seen (Fig. 6.73)

3. Time Course of Fibrillar Granule Appearance

The first fibrillar granules may appear when the oocyte is only perhaps 20 μm in diameter, and sometimes prior to its entry into the mesogloea. They usually appear before compound yolk granules are present in any number, although lipid droplets and glycogen are accumulated from the very earliest stages. In small oocytes, the fibrillar granules themselves are usually very small (Fig. 6.74), often only 0.2-0.5 μm in diameter, but may exhibit a range of fibril packing densities.

As the oocyte enlarges, the fibrillar granules increase in size and number, and, at an oocyte diameter of perhaps 50 μm, become the dominant cytoplasmic inclusion (Fig. 6.67). Fibrillar granules of all densities may still be found intermingled at this stage. At about this time, compound yolk granule production increases, and they become more common in

the cytoplasm. It is not clear whether fibrillar granule production slows after this stage, or whether it is merely overtaken and eventually swamped by compound yolk formation, but by the time the oocyte reaches about 80 μm diameter, compound yolk granules predominate. By the time the oocyte is fully grown, fibrillar granules are usually restricted to a peripheral layer extending perhaps 10 μm from the oocyte surface; the central region is largely filled with compound granules (Fig. 6.75). In large oocytes, most, but not all, of the fibrillar granules are closely packed.

4. Association with Golgi Complexes

Throughout vitellogenesis, the oocyte contains large quantities of rough endoplasmic reticulum, and large numbers of Golgi complexes. Especially during the early part of vitellogenesis, a high proportion of Golgi complexes are associated with fibrillar granules. Such complexes are usually made up of stacks of 7-10 flattened cisternae, which are often curved. Several such stacks may be grouped together to form a complex (Fig. 6.76). Usually the cisternae are narrow and closely packed together, although their ends may be dilated. One or two cisternae of endoplasmic reticulum are invariably associated with these complexes. Very often, the ER at least partially encircles the complex (Fig. 6.77), and numerous small vesicles are found between the two. The ER and outer Golgi cisternae contain a wispy, finely fibrillar material of low electron density. The more central Golgi cisternae tend to be narrower and enclose a more dense material. The cisternal membranes progessively increase in thickness across the stack, from approximately 7 nm at the periphery to 10 nm at the centre (Fig. 6.66).

The enclosed central area of the complex contains numerous small vesicles, and fibrillar granules of various sizes. Rarely, lipid droplets and compound yolk granules may also be found (Fig. 6.78). The fibrillar granules vary greatly in size and packing density, but usually contain clearly defined fibrils. Often vesicles can be seen apparently fusing with the granules, and sometimes whole lengths of the innermost Golgi cisternae appear to fuse also, such that there is membrane continuity between the Golgi and the granule (Figs. 6.79, 6.80). Golgi complexes were never observed in close association with large loosely packed granules in which the fibrils were poorly defined.

A less direct association between Golgi and fibrillar granules was also sometimes observed. Golgi complexes similar to those described above are sometimes seen associated with membrane-bound vesicles some 250 nm in diameter, containing a flocculent material of moderate electron density (Fig. 6.81). Vesicles of similar size and similar, slightly greater, density may also be found associated with nearby fibrillar granules.

Fibrillar granules may also be found associated with vesicles when not in the vicinity of a Golgi complex. Sometimes the membrane around these granules is ruffled, and it seems possible that fusion between the vesicles and granules may occur (Fig. 6.82). Fig. 6.83 shows a fibrillar granule with a protuberance at one end. This protuberance contains non-fibrillar granular material, identical to that seen inside a nearby small vesicle. Again, it seems possible that fusion of granules with small vesicles can take place in these situations.

DISCUSSION

In *A. fragacea* oocytes, a number of forms of membrane-bound body are found which, while varying considerably in appearance, all contain material with a fibrillar substructure. On the findings of the present study, the various forms all appear to represent variations of a single granule type, and all have been termed fibrillar granules.

Fibrillar granules have been reported from anthozoan oocytes on several previous occasions. Clark and Dewel (1974) described two types of inclusion from oocytes of the sea anemone *Metridium senile*. The first type were termed rod-shaped inclusions, and are composed of an electron dense material which occasionally exhibits a distinct crystalline lattice. The second type of inclusion is membrane-bound and contains large quantities of fibrous material. From their published micrographs, both types closely resemble the fibrillar granules of the present study, and may also represent different forms of the same structure. Dewel and Clark (1974) mentioned that both membrane and nonmembrane-bound fibrous bodies were also common in oocytes of the sea anemone *Bunodosoma cavernata*. Fibrillar granules lacking a limiting membrane would appear to represent only a very small fraction of the total in *A. fragacea* oocytes, however. Schäfer and Schmidt (1980) and Schäfer (1983) have described fibrillar paracrystalline inclusions, which appear to correspond to the fibrillar granules of the present study, from a number of anthozoan oocytes. They state that these inclusions occur in oocytes of Madreporaria, Actiniaria, Zoantharia and Antipatharia and suggest that they exhibit characteristic forms in particular taxonomic groups. For example, the inclusions tend to be round

or polygonal in scleractinians, but more elongate in actiniarians. The fibrils are arranged in parallel in Madreporaria, Actiniaria and Zoantharia, but form concentric rings in Antipatharia. They also describe some forms found in particular species. In *Bunodeopsis strumosa*, for example, the inclusions tend to have a central electron dense core, while in *Gerardia savaglia*, the periphery of the inclusion is more dense than the centre. They also describe the individual fibrils in general as resembling asterisks when seen in cross section. Given the tremendous variation in the appearance of these granules encountered in the present study, even within single, oocytes, it would appear that caution is indicated when making generalizations about granule form in particular species or groups, although consistent differences may well exist. Whether these differences are of any taxonomic significance seems doubtful, especially considering that fibrillar granules are abundant in *A. fragacea* oocytes, but are apparently lacking in those of *A. equina*.

Formation of Fibrillar Granules

The evidence obtained during the present study does not all point to a single mode of formation for all fibrillar granules, but rather suggests that several processes may operate, possibly to varying degrees in different granules.

There would appear to be strong evidence implicating the Golgi apparatus in the production of fibrillar granules. Granules were often found associated with Golgi complexes, and in some instances the association seemed so intimate that it seemed unlikely to be merely fortuitous. Membrane continuity between the inner Golgi cisternae and the granule was frequently observed, and small vesicles, apparently of Golgi

origin, often appeared to fuse with the granule during the association. The Golgi complexes found associated with fibrillar granules had a characteristic appearance. They were always associated with one or sometimes two cisternae of endoplasmic reticulum, which was not often the case with other Golgi complexes, and small vesicles were often observed between the ER and the outer Golgi cisternae. The evidence obtained in the present study is consistent with the following sequence of events leading to the formation of fibrillar granules by the Golgi apparatus:

i. Precursor material, presumably protein, is synthesized on the RER and transported through the lumen of the ER to the vicinity of the Golgi complex.

ii. Small vesicles budding off the ER transfer the precursor material to the outer or forming face of the Golgi complex.

iii. This material passes across the complex to the inner or maturing face, possibly undergoing modification as it does so.

iv. Small vesicles detach from the innermost Golgi cisternae and fuse to form small granules, within which the fibrillar substructure becomes apparent. Some longer lengths of the inner cisternae may also fuse with the granule.

v. The forming granule enlarges by fusion of further small vesicles.

Such a scheme is broadly consistent with current views of Golgi function in many cell types (e.g Göldfischer, 1982; Alberts *et al*, 1983) and is similar to that proposed by Beams and Kessel (1983) for the formation of one type of yolk in oocytes of a trachyline medusa.

It also seems possible that small vesicles can fuse with fibrillar granules which are not closely associated with Golgi complexes. It may be that the non-fibrillar component often seen at the ends of large granules represents material which has recently been added in this way. There is also evidence that fibrillar granules can enlarge by fusion with each other. In small granules, all the fibrils are usually aligned along a single axis, while larger granules often appear to be composed of several bundles of fibrils, each aligned along a different axis. This situation can readily be explained by granule fusion. Additionally, several instances of what appear to be granules in the process of fusing with each other were observed. Fusion of yolk bodies was also reported by Beams and Kessel (1983).

A major difficulty surrounding the formation of fibrillar granules is the extent to which the different densities and forms of fibril packing found in different granules represent a developmental series. A series could be proposed, starting from small, non-fibrillar vesicles which fuse to form larger granules, within which a fibrillar arrangement develops. The granule contents might increase in density and regularity until mature, highly ordered, closely packed granules were produced. Schäfer (1983) has proposed that fibrillar granules in Anthozoa are formed by the conversion of non-fibrillar material into the final ordered fibrillar form. He suggested that Golgi complexes produce primary, osmiophilic yolk granules, which are non-fibrillar, and which give rise to all the various forms of yolk. Especially early during vitellogenesis, the contents of these primary granules crystallize to form fibrillar paracrystalline inclusions. However, during the present study, no evidence that fibrillar granules derive from

a dense precursor material was obtained.

Another difficulty is that morphological studies may not provide a clear indication of the direction of flow of structures along a developmental pathway. It may be that loosely packed granules actually derive from more closely packed ones rather than the reverse, and could represent a stage in their breakdown or utilization. Loosely packed granules are often large, and may have a discontinuous limiting membrane. Granules which presumably have recently been formed, such as those in small oocytes or those still associated with Golgi complexes, on the other hand, are usually small, have intact membranes and contain well-defined fibrils. From the present study, it is not possible to resolve the status of the loosely packed granules. A scheme showing some of the possible interactions during fibrillar granule formation is given in Diagram 8.

The function of the fibrillar granules is also uncertain. There is some evidence that they may contribute to the formation of low density granules which may discharge to the outside of larger oocytes (see Section H of this chapter). During embryonic development, fibrillar granules also discharge to the outside, and their numbers drop to zero by the planula stage (see Chapter 13). However, it is not clear whether they are all discharged in this way, or what function this discharge performs.

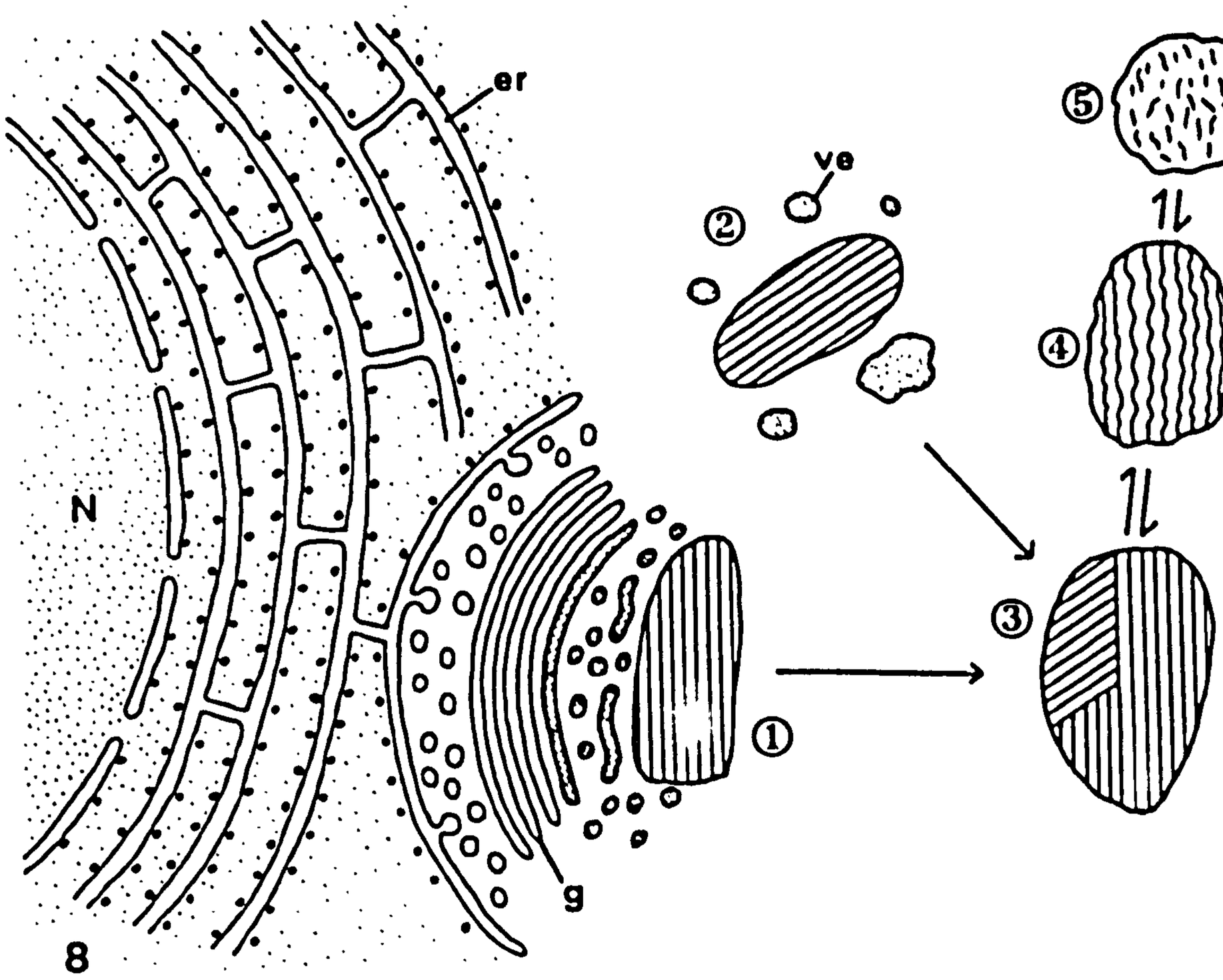


Diagram 8.

Diagram illustrating possible steps leading to the formation of fibrillar granules. Many fibrillar granules (1) appear to be formed in close association with Golgi complexes, which in turn are associated with rough endoplasmic reticulum. Other fibrillar granules (2) may form or grow by the addition of small vesicles not obviously associated with Golgi complexes. Granules from either source may fuse to produce larger granules (3), in which the fibrils are not all aligned along a single axis. Regular, closely packed fibrillar granules appear to be related to less regular, loosely packed granules (4) and granules in which fibrils are only barely discernible (5), but the nature of this relationship is uncertain.

Chapter 6, Section E: Compound Yolk Granules (CYG)

RESULTS

The term compound yolk granule has been applied to a diverse class of roughly spherical bodies which accumulate in the growing oocyte and which share the common characteristic of containing one or many spherical electron lucent inclusions embedded in an electron dense matrix. Very small numbers of granules which resemble small compound yolk granules may be found in small oocytes still within the endoderm. However, large scale production of these granules does not normally occur until after entry into the mesogloea is complete, and in most cases begins later than fibrillar granule production (Fig. 6.84). But while fibrillar granule production reaches a peak relatively early, and declines in oocytes above perhaps 80 μm in diameter, compound yolk granule formation, once begun, continues throughout oocyte growth, and these granules become the dominant cytoplasmic inclusion in the fully grown oocyte (Fig. 6.85). Compound yolk granules change markedly in appearance as oocyte growth proceeds. The granules found in large oocytes, which may represent the mature or definitive form, will be described first, before going on to consider the granules found in smaller oocytes, which may represent intermediate stages.

1. Compound Yolk Granules in Large Oocytes

In oocytes over about 100 μm in diameter, the most common form of CYG found in the cytoplasm resembles that shown in Fig. 6.86. These granules average some 1.5 μm in diameter, are roughly spherical in shape and have a generally smooth outline. They are surrounded by a close-fitting, 10 nm wide

trilaminar limiting membrane, and contain a highly electron dense matrix material in which are embedded numerous spherical electron lucent inclusions of various sizes. These inclusions are not membrane bound, although some appear to be surrounded by a thin layer of very dense material some 5 nm thick. In density and overall appearance, the inclusions resemble the lipid droplets which are common in the ooplasm, and which will be described in the next section of this chapter. Usually the inclusions range in size from 70-400 nm, and appear to be randomly distributed through the dense matrix. The matrix material itself appears finely granular but otherwise homogeneous.

While this may be taken as the most typical form of compound yolk granule, many granules deviate from this appearance in one or several respects. Much of the observed variation can be considered under the following three headings:

a) Variation in the ratio of inclusions to matrix material.

b) Variation in the size and distribution of the inclusions.

c) Presence of membranous and other structures within the matrix material.

Taking these in turn:

a) Some granules appear to be packed virtually full of electron lucent inclusions such that the amount of dense material is considerably reduced (Fig. 6.87). In others, the relative volume of the inclusions may be reduced.

b) Most granules contain numerous small inclusions, but some contain relatively few, larger inclusions (Fig. 6.88). These larger inclusions are often situated at the periphery of the granule, and may cause the limiting membrane to bulge outwards

over them (Fig. 6.89). Often, the inclusions appear to be distributed non-uniformly through the dense matrix. Thus, relatively large areas of matrix material may be found between clusters of closely packed inclusions (Fig. 6.90). Commonly, the matrix forms a band around the periphery of the granule, while the inclusions are concentrated towards the centre (Fig. 6.91).

c) Many granules contain what appear to be membranous elements in the matrix. The form of these elements may vary considerably. Most commonly they appear as stacks of parallel short lengths of membranous material scattered throughout the matrix of the granule (Fig. 6.92). Sometimes the stacks may be curved or whorled to encircle one or several inclusions (Fig. 6.93), and may be tightly packed, with a spacing of some 20 nm, to resemble the myelin found around vertebrate axons (Fig. 6.94).

Less commonly, straight-sided inclusions, similar to those found in residual body-like inclusions, are found within compound yolk granules. These may be slender and needle-like (Fig. 6.95) or squat (Fig. 6.96). They are generally of lower electron density than the spherical inclusions.

2. Association with Other Structures

Large apparently fully-formed compound yolk granules are very often found associated with usually a single annulate lamella (Figs. 6.97, 6.98). These usually curve around part of the surface of the granule, at a distance of some 60 nm from it, but never completely encircle granules. Less commonly, a cisterna of endoplasmic reticulum, without pore complexes, is found immediately outside the annulate lamella (Fig. 6.97). Cisternae lacking pore complexes were not found next to the

granules, however.

Small membrane bound vesicles and small lipid droplets were also seen close to compound yolk granules.

3. Compound Yolk Granules in Smaller Oocytes

Large compound yolk granules as described above are rarely found in the cytoplasm of oocytes smaller than perhaps 70 μm in diameter. In these smaller oocytes, a variety of smaller granules and vesicles are found, some of which appear to be clearly related to the larger granules, and all of which may be involved in compound granule formation. These various types of inclusion will be described, starting with the apparently simpler forms and moving to the larger and more complex types.

a) Many smaller oocytes contain small vesicles with highly electron dense, finely granular contents. They average some 100 nm in diameter, and most are roughly spherical (Fig. 6.99) although some may be elongate and more irregular in shape (Fig. 6.100).

b) Small membrane-bound granules with vesicular contents are also very commonly found. They appear to be of two rather different types. The first type contains large numbers of small, spherical vesicles (each some 25 nm in diameter), closely but irregularly packed. Each small vesicle consists of an outer dense coat, which is thinner than a unit membrane and does not show a trilaminar structure, surrounding a less dense interior (Fig. 6.101). Sometimes lipid inclusions and membrane whorls are found within these granules (Fig. 6.102).

The second type of granule also contains numerous small vesicles of similar size and appearance to those described above, but in this case, the vesicles are all of precisely

the same size, and they show a degree of regular arrangement. The vesicles tend to be aligned in roughly straight rows, and neighbouring rows are half a diameter out of phase, to give a hexagonal, close-packing arrangement (Fig. 6.103). Some of these granules contain regions of uniformly dense material with no vesicular substructure (Fig. 6.104), and, less commonly, they may contain a few spherical, electron-lucent inclusions like those found in large compound granules (Figs. 6.105, 6.106).

c) Many smaller oocytes contain a range of membranous tubules and cisternae. Often these are arranged in parallel stacks, resembling small Golgi complexes, but less orderly and lacking recognizable forming or maturing faces. These structures are often associated with large numbers of small dense vesicles of both types described above (Figs. 6.107, 6.108).

d) Small membrane-bound granules with contents of only moderate electron density are also found. The contents may be finely or coarsely granular, and the limiting membrane may appear loosely fitting. Very often these granules also contain stacks or whorls of dense, closely-packed membranous material (Figs. 6.109, 6.110). They may also contain one or more electron lucent lipidic inclusions.

e) Small lipid droplets surrounded by 1-3 concentric layers of trilaminar membrane are also found. These are usually 100-200 nm in diameter, and they may be smooth or scalloped in outline (Fig. 6.111).

f) One of the most common granules found in oocytes between 50 and 70 μm, consists of a single lipid droplet or inclusion embedded in dense material. The amount of dense material may

vary from a thin rim to an extensive layer, and it may be homogeneous, granular or have a vesicular substructure, and nearly always contains membranous whorls. The lipid inclusion is very often located eccentrically within the granule (Figs. 6.112 -6.114). Similar, but slightly larger granules, may contain more than one lipid inclusion, and may contain several membranous whorls (Fig. 6.115). These granules resemble smaller versions of the fully-formed compound granules described earlier, and are also sometimes associated with annulate lamellae (Fig. 6.116).

g) Some small oocytes contain extensive double-membrane sheets or flattened cisternae, which may be arranged in irregular whorls, and may surround small lipid droplets and dense vesicles (Fig. 6.117). Some whorls appear more regular and compact, and may represent a later stage in the formation and condensation of these structures.

h) Rarely, atypical compound yolk granules are found, especially in rather larger growing oocytes. These are usually spherical and smooth in outline, and contain a granular matrix material of only moderate electron density with small lipid inclusions. Membranous whorls are not found among the matrix material, the proportion of inclusions to matrix is lower and the overall density of these granules is lower than the typical compound yolk granule (Fig. 6.118).

4. <u>Vesicular and Satellite Granules</u>

Two other types of granule are found in the cytoplasm, especially of larger oocytes, which may be related to the compound yolk granules.

Vesicular granules are membrane-bound bodies containing an electron dense material which appears to consist of numerous

small spherical veiscles, similar to that found in the smaller granules described in Section 3b, above. The granules are 1-2 μm in diameter and are usually roughly spherical, although some are less regular (Fig. 6.119). In some cases, the small vesicles are all of roughly similar size, averaging 25 nm, but often rather larger vesicles are interspersed with them (Figs. 6.120, 6.121). The small vesicles appear to be of moderate electron density, while the larger ones seem much less dense. Since the smallest vesicles are smaller than the thickness of the section, their apparently greater density may be artefactual. The larger vesicles closely resemble the electron lucent inclusions found in compound yolk granules, and many vesicular granules also contain membranous elements (Fig. 6.121) and regions of homogeneous dense material, also reminiscent of compound granules (Fig. 6.122). Dumb-bell shaped vesicular granules are sometimes seen (Fig. 6.123), which may arise by the fusion of smaller granules. Rarely, granules consisting of rather larger vesicles occur (Fig. 6.124). Most of these vesicles are approximately 50 nm in diameter, and some are considerably larger. These granules appear intermediate in form between the typical vesicular granules and compound yolk granules. Vesicular granules account for some 3% of compound yolk granules in large oocytes.

Satellite granules are also roughly spherical, membrane-bound bodies between 1 and 2 μm in diameter. They are even less common than vesicular granules, and their numbers are hard to estimate. They contain coarsely granular material of moderate electron density, in which may be embedded electron lucent inclusions and membranous elements (Figs. 6.125, 6.126). These inclusions are usually clustered near the periphery of

the granule rather than scattered through it, and some may distort the outline of the granule. Characteristically, these granules are ringed by usually a single row of small vesicles averaging 80 nm in diameter. These contain a finely granular material of rather variable electron density, but which is usually more dense than the contents of the central granule. The outer vesicles are usually separated from the central granule by a gap of 20 nm or more, but occasionally the gap is reduced. It is possible that fusion or budding off of these vesicles takes place.

DISCUSSION

Bodies termed 'Komplexdotter' have been observed in the oocytes of the hydrozoans *Corydendrium parasiticum* and *Eudendrium armatum* by Glatzer (1971) and Wasserthal (1973) respectively, and resemble, but are not identical to, the compound yolk granules of *A. fragacea*. Similar structures are not found in all Hydrozoa, however. They have not been found in oocytes of *Hydra* (Stagni and Lucchi, 1964), *Spirocodon saltatrix* (Kawaguti and Ogosawara, 1967) or those of the trachyline medusa examined by Kessel (1968a;Beams and Kessel, 1983). The yolk granules of *Sertularella polyzonias* are homogeneous (Honegger, 1980). Schäfer and Schmidt (1980) and Schäfer (1983) report that heterogeneous yolk or 'Komplexdottergranula' of various types occurs in oocytes of a wide range of anthozoan species.

The compound yolk granules of *A. fragacea* bear at least a superficial close resemblance to the vitelline platelets found in the archeocytes of gemmules of freshwater sponges such as *Ephyhydatia mulleri* (Tessenow, 1969). According to

Tessenow, these platelets are lens-shaped and consist of a protein matrix in which are embedded numbers of lipid droplets. Ribonucleoprotein is located at the surface of the platelet, and the platelet is surrounded by numerous membranes. However, De Vos (1971) working with *Ephyhydatia fluviatilis* has also found mitochondria, glycogen and ribosomes in early and fully-formed platelets, and concludes that they represent the remains of trophocytes which have been phagocytosed by the archeocytes. Structures apparently identical to *A. fragacea* compound yolk granules occur in oocytes of marine sponges of the genus *Verongia* (Gallissian and Vacelet, 1976). Since no signs of phagocytosis were observed in these oocytes, the authors concluded that these yolk platelets must be elaborated within the ooplasm. Unfortunately, they could not establish the mechanism by which they were formed. The finding of structures similar to compound yolk granules in poriferan gemmules and oocytes is of interest, but its significance is hard to assess.

Schäfer (1983) distinguishes four categories of compound yolk granules in anthozoan oocytes, which he designates Types A1, A2, B1 and B2. Type A1 granules consist of a dense matrix in which are embedded a relatively small number of small spherical inclusions of lower density. Superficially, these granules resemble many vesicular granules found in *A. fragacea*, which contain a few larger vesicles. However, he does not report the presence of vesicles within the matrix, and none are discernible in his published micrographs. Type A2 granules are similar to those of Type A1, but include regions of a crystalline material. Crystalline inclusions were not found in any compound granules in *A. fragacea* oocytes, but were observed in those of *Cereus pedunculatus* (see Chapter 10).

Type B1 granules also contain a dense matrix, in which are embedded a small number, usually one, of large spherical lipidic inclusions, while Type B2 granules contain numerous smaller inclusions. Granules corresponding to both of these categories made up the majority in *A. fragacea* oocytes However, Schäfer does not mention or illustrate the presence of membranous stacks and whorls in any of these types, although they were frequently found in *A. fragacea* granules during the present study, and are also common in *Cereus peduncularus* and *Tealia felina* (see Chapter 10).

Schäfer and Schmidt (1980) propose a scheme for the formation of these different granules, which is further elaborated by Schäfer (1983). Briefly, they suggest that Golgi complexes give rise to small vesicles containing an osmiophilic primary yolk material, which may fuse to yield primary yolk granules. Some of these may transform into granules of Type A1, which in turn may convert to Type A2 by the crystallization of some of the matrix. Other primary yolk granules, possibly of a different type, may fuse with lipid droplets to form Type B granules. If they fuse with one large lipid droplet, and little subsequent dispersion occurs, they produce Type B1 granules. If they fuse with several droplets or considerable dispersion takes place, then Type B2 granules are formed.

On the evidence obtained during the present study, it is not clear that the compound yolk granules in *A. fragacea* oocytes can easily be subdivided into discrete categories. Small granules corresponding to Schäfer's Type B1 were very common in smaller oocytes, while larger granules approximating to Type B2 predominated in larger oocytes. It seems possible that

B1 granules could represent an intermediate stage in the formation of those of Type B2. Similarly, intermediate forms were found between vesicular granules, which may correspond to Type A1, and Type B2 granules. It may be that compound yolk granules in *A. fragacea* present a continuum of forms, which may be subdivided arbitrarily for convenience but which are all expressions of a similar underlying structure.

The modes of compound yolk granule formation proposed by Schafer would seem to be a simplification of the situation in *A. fragacea*. Given the range of final and intermediate forms observed, it seems likely that a number of processes may operate. Based largely on observations of smaller oocytes, these might include the following:

i. Small, membrane bound vesicles arise in the cytoplasm. Their contents may be dense and homogeneous, or less dense and granular or vesicular. While these vesicles may well be Golgi derived, no clear association between them and Golgi complexes was observed.

ii. Membranous stacks and whorls can form within small vesicles and around the circumference of lipid droplets.

iii. Lipid droplets, often with surrounding membranes, can fuse with all the forms of small vesicles to form small granules with one or more lipid inclusions.

iv. These small granules can fuse with each other to form larger and more complex granules.

v. Rearrangement of the contents of these granules may also occur. Many compound yolk granules in small oocytes contain myelin-like membrane whorls, while in larger oocytes, parallel stacks of relatively straight membranes are more common.

The lipid inclusions are generally smaller and more numerous in granules from large oocytes than from those in small, so a splitting and dispersion of the inclusions may well take place.

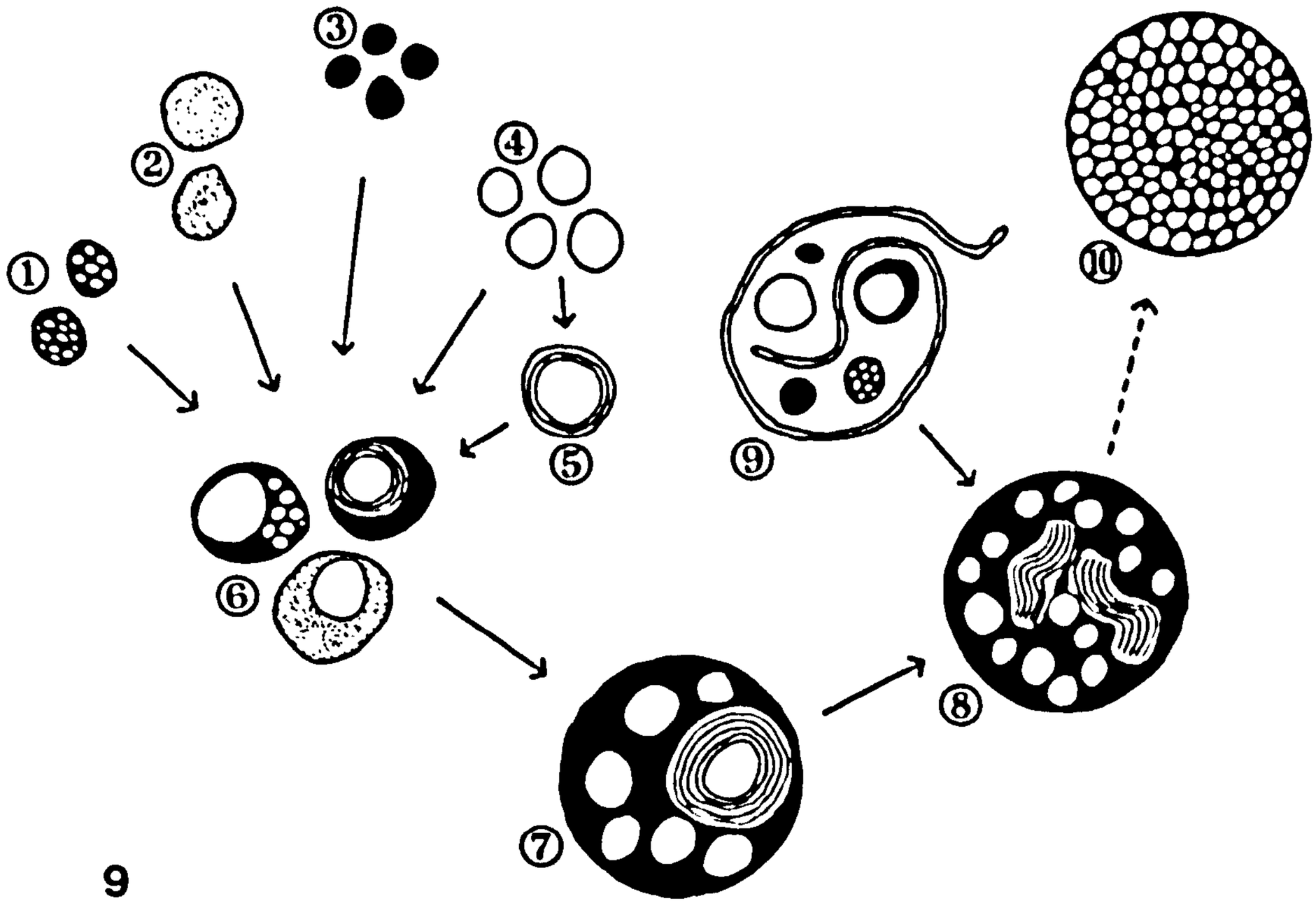
vi. A small proportion of large granules may be formed more directly by the condensation of extensive membranous sheets around small vesicles and lipid droplets.

The possible steps in compound yolk granule formation are summarized in Diagram 9. Since large and complex granules are rarely found in small oocytes, it seems likely that steps (iv) and (v) of the above scheme only take place in oocytes larger than perhaps 70 μm.

These findings are broadly consistent with the proposals of Schäfer (1983). The above scheme, however, allows for greater plasticity, which, in view of the range of granule types found in both small and fully grown oocytes, seems essential. A major difference is that no well-defined precursor material corresponding to Schäfer's primary osmiophilic yolk was ever observed in *A. fragacea* oocytes.

The status of the vesicular granules remains unclear. Granules of intermediate appearance between these and compound granules were occasionally seen in *A. fragacea*, and are quite common in *Cereus* oocytes (see Chapter 10). It therefore seems possible that vesicular granules are the result of a more complete fragmentation and dispersal of the lipidic inclusions than occurs in other compound yolk granules.

Finally, it should perhaps be noted that the cytoplasm of vitellogenic *A. fragacea* oocytes contains a vast array of small, membrane bound vesicles of various sorts. The function



9

Diagram 9.

Possible steps in the formation of compound yolk granules. Early during vitellogenesis, various small inclusions appear in the ooplasm. These include membrane-bound vesicles containing vesicular (1), moderately dense (2) and highly dense (3) material and lipid droplets (4), some of which acquire a coating of membranous material (5). These inclusions may fuse with each other in various proportions, to form a heterogeneous population of early compound yolk granules (6), which accumulate in relatively small oocytes. In larger oocytes, these in turn fuse to form larger granules (7), which undergo reorganization of the membranous elements and dispersion of the lipidic inclusions to produce the final compound yolk granule (8). Some granules may be formed by the condensation of double-membrane whorls around small inclusions (9). Vesicular granules (10) may be produced from compound yolk granules by the further dispersion of the lipidic inclusions.

of these vesicles is usually unknown, but they may be highly significant. The importance of such small and diverse components is always likely to be undervalued in morphological studies of this sort.

Chapter 6, Section F: Glycogen and Lipid Droplets

RESULTS

Glycogen and lipid droplets are the first reserve materials to be accumulated by the growing oocyte. Both are usually present, albeit in small quantities, in the smallest oocytes, and their amounts often increase long before vitellogenesis, in the sense of the formation of more specific yolk bodies, begins.

1. Glycogen

In the very smallest oocytes, glycogen is usually found as numerous small deposits scattered throughout the cytoplasm (see Chapters 4 and 5). As the oocytes grow within the endoderm, the glycogen tends to form a smaller number of larger deposits (Fig. 6.127). These larger deposits are very often associated with lipid droplets, as will be discussed later in this section, and may contain principally either β- or α-glycogen (Fig. 6.128). During the period shortly before and after oocyte entry into the mesogloea, the quantities of glycogen increase and it may become a very significant component of the ooplasm. The large deposits found at this stage very often are situated near the surface of the oocyte (Fig. 6.129).

After entry into the mesogloea and into early vitellogenesis, the build up of glycogen reserves within the oocyte continues, and very large deposits can be formed. Although these can occur anywhere in the ooplasm, they are most often found either next to the nucleus (Fig. 6.130) or near the periphery of the oocyte (Fig. 6.131), and they may reach 10 µm in diameter. Later in vitellogenesis, however, once compound

yolk synthesis becomes significant, glycogen deposition does not keep pace with oocyte growth, and it progressively becomes a less dominant feature of the ooplasm. During mid-vitellogenesis, large deposits are still found, however, but they are often broken up by small islands of cytoplasm. These are usually 0.3-0.5 μm in diameter, and contain mainly ribosomes, although mitochondria, small yolk granules and dense bodies can also occur within them (Fig. 6.132). Extensive clear areas, often limited by a thin layer of dense material, may also be found (Fig. 6.133), which may represent the remnants of lipid material previously associated with the glycogen (see below).

Throughout oogenesis, there is a tendency for smaller glycogen deposits to contain a higher proportion of glycogen in the β-formation than larger deposits. The typical rosettes of α-glycogen found in larger deposits tend still to be smaller than the particles found in the bases of the gonad epithelial cells.

In oocytes smaller than perhaps 30 μm in diameter, glycogen is very often closely associated with the various forms of nuage material (see Chapters 4 and 5). In larger oocytes, the association is less obvious, but is still occasionally observed (see Section G of this chapter).

Occasionally in anemones from the Wembury population, but very commonly in those from Shaldon, masses of fibrils were found within glycogen deposits (Fig. 6.134). Each fibril is some 12 nm in diameter, and they are usually grouped in roughly parallel bundles at the centres of the deposits. Some more randomly arranged fibrils were also found in the outer regions of deposits, however (Fig. 6.135), and in a few cases the fibrils were dispersed randomly among the

glycogen particles (Fig. 6.136). Such filaments were never observed elsewhere within the oocytes, and their significance is unclear.

As the oocytes approach their final size, the remaining glycogen deposits appear to break up. Recently spawned eggs contain only small quantities of glycogen, mainly as small, scattered deposits.

Mention must be made of the unusual appearance of glycogen deposits in gonad samples subjected to a tertiary fixation with aqueous uranyl acetate. The characteristic appearance of the glycogen was dramatically altered by this procedure. β -granules and α-rosettes could no longer be distinguished, and at low magnification the deposits appear as areas of scattered finely granular material, with a very low overall electron density (Fig. 6.137). At high magnification, they were seen to consist of an open lacework of amorphous electron dense material, among which small dense granules were irregularly distributed (Fig. 6.138). The effect of uranyl acetate on the appearance of glycogen has been reported by previous authors (see Discussion).

2. Lipid Droplets

Even the smallest oocytes contain lipid droplets. These occasionally occur singly, but often they are found as small groups, which are usually associated with glycogen (Figs. 6.127, 6.128). Two classes of lipid droplet can be distinguished in small oocytes. The most common type is homogeneous and of moderate electron density. They range in size from 0.25 μm to 1.0 μm, and some of the larger droplets may be dumb-bell shaped, having perhaps arisen by the

coalescence of smaller droplets (Fig. 6.139). These droplets are identical in appearance to the droplets found in the basal regions of the gonad epithelial cells. Occasionally, there appears to be an extremely thin layer of dense material around some of the droplets (Fig. 6.139), but usually this is not the case and they make close contact with the surrounding material, which is very often glycogen (Fig. 6.140).

The second type of droplet is of very low electron density and may appear to be completely empty. They are of similar size to the first type, and are also usually associated with glycogen (Figs. 6.139, 6.140). They are usually surrounded by a thin layer of dense material which is thinner than a conventional unit membrane and has no trilaminar substructure (Fig. 6.141). These droplets may have a mottled or otherwise uneven appearance, which may be due to extraction of components during tissue processing. They may also be closely associated or continuous with the first type of droplet (Figs. 6.140, 6.142).

During the endodermal and pre-vitellogenic phase of oocyte growth, the amounts of stored lipid and glycogen increase at about the same rate, and large mixed deposits may form. Once vitellogenesis gets under way, however, the rate of glycogen accumulation appears to slow, but the quantities of lipid continue to increase rapidly. From this stage onwards, the association between glycogen and lipid becomes much less pronounced. Groups of lipid droplets appear throughout the ooplasm, and are only rarely found in contact with glycogen deposits. These unassociated droplets are all of the first type described above (Fig. 6.143), but may reach a size of 2 μm in large oocytes.

In larger oocytes, over 80 µm in diameter, lipid droplets are often found associated with rough endoplasmic reticulum (Fig. 6.144). Commonly, the droplets are surrounded by one or several cisternae which may cover most or all of their surface. The innermost cisterna is usually separated from the droplet by a narrow and fairly constant gap of some 50 nm, and may be liberally studded with ribosomes on both aspects. This association is more common with large lipid droplets than small ones and also appears more precise. The cisternae around small droplets are often interrupted by numerous fenestrations, and may only cover part of the droplet (Fig. 6.144) while those around large droplets are usually more complete (Figs. 6.145, 6.146). Large droplets may be surrounded by up to ten cisternae (Fig. 6.147). Pore complexes have not been observed in cisternae around lipid droplets, and no association between lipid droplets and smooth endoplasmic reticulum was observed.

DISCUSSION

Anderson (1974) defines yolk as "Any substance(s) nutritive or informational, found within the egg, that is utilized subsequent to the egg's activation for the support of embryogenesis." This definition would in many cases include material other than the proteinaceous reserve bodies commonly referred to as yolk. More recently, Adiyodi and Adiyodi (1983a) state " The recent tendency in some quarters to restrict the use of the term 'vitellogenesis' to formation of only the proteinaceous type of yolk is to be deprecated." According to these authors, the lipid and glycogen reserves which accumulate during oocyte growth in *A. fragacea* should be regarded as yolk, and, since these are present in the

earliest rocognizable oocytes, such tiny oocytes could already be considered to be undergoing vitellogenesis. Whether this view will be universally accepted or not, glycogen and lipid form an important reserve in oocytes of many species, and have perhaps received less research attention than they warrant (Anderson, 1974).

Glycogen is a branched polymer of glucose molecules, primarily linked together by 1-6 α glycosidic linkages, and is an extremely common storage product in many animal cells. In many cases, glycogen is arranged in large rosettes, the so-called α -particles, which are made up of smaller, granular subunits known as β -particles (De Robertis *et al*, 1975). In *A. fragacea*, most deposits contain a mixture of the two forms in varying proportions. Glycogen deposits generally, including those of *A. fragacea*, appear to be able to enlarge or reduce in size with no obvious intervention by other cellular organelles. It is thought that the enzymes responsible for glycogen synthesis and breakdown, principally glycogen synthase and glycogen phosphorylase respectively, are bound to the surface of the particles (Alberts *et al*, 1983).
The apparent fall in glycogen levels seen in the later stages of oogenesis in *A. fragacea* may indicate its utilization either as an energy source, or, perhaps more likely, in the synthesis of other oocyte components. Its use prior to fertilization and egg activation might be argued to compromise the status of at least a proportion of the glycogen as yolk under Anderson's definition (see above).

Vye and Fischman (1970) investigated the effect of uranyl acetate used as a fixative or *en bloc* stain on the morphology of particulate glycogen in a range of mammalian and avian tissues. The effect varied in the different tissues, but was

broadly similar to that seen in the present study. They concluded that the use of uranyl acetate in this way should not be regarded as an innocuous procedure.

Lipid droplets of relatively low electron density in conventionally prepared sections are generally composed of neutral lipid in the form of triglycerides. They are a very common storage product in numerous cell types, and constitute a reserve of fatty acid molecules which are important in a number of major metabolic pathways. In many cases, the insoluble tryglycerides coalesce in the cytosol to form large spherical droplets, a process which again may occur without relationship to any known organelles (Norrevang, 1968). No very small lipid droplets were found in *A. fragacea* oocytes, suggesting that new lipid material might be added directly to the surface of existing droplets rather than forming tiny droplets which later coalesce.

Schäfer (1983) reports a close association between lipid droplets and narrow tubular elements of smooth endoplasmic reticulum in anthozoan oocytes generally. No such association was apparent at any stage in oocytes of *A. fragacea* or of other species studied here (see Chapter 10). An association was noted, however, between lipid droplets and glycogen, especially in smaller oocytes, and also between lipid droplets and flattened cisternae of rough endoplasmic reticulum in larger oocytes. This second association was more common and apparently more highly developed with large lipid droplets where the components seemed too precisely arranged for it to be merely fortuitous, although its functional significance is not clear. A very similar association was reported and illustrated for oocytes of the ophiuroid *Ophioderma panamensis* by Kessel

(1968b). He shows whorls of flattened ER cisternae arranged around lipid droplets in an identical manner to that seen in *A. fragacea*. He also concluded that the significance of this association was unknown, but pointed to some biochemical evidence that in some other cell types, notably mammalian liver cells, the enzymes responsible for the formation of triglycerides are bound to the membranes of both smooth and rough ER (Stein and Stein, 1967). In this situation, the aggregation of triglyceride molecules into visible droplets takes place within the ER cisternae. However, no lipid droplets were observed within ER cisternae either by Kessel or in the present study. Schäfer (1983) reports Kessel (1968b) as finding that these lipid droplets were associated with preponderantly smooth ER, in a manner comparable to his own findings with anthozoan oocytes. However, Kessel states that ribosomes were distributed sparsely between and on the ER membranes involved. He points out that the number of ribosomes associated with ER in the ophiuroid oocyte generally is lower than in pancreatic cells, for example, even though the ER is implicated in proteid yolk production. The form of the ER around the lipid droplets is clearly seen as whorled, flattened cisternae, rather than the tubular form characteristic of smooth ER, and as described by Schäfer. Thus it may be that the ER associated with lipid droplets in *Ophioderma* could more correctly be described as rough ER, and may bear a closer similarity to the situation found in *A. fragacea* than to that described by Schäfer (1983).

Glycogen and lipid droplets have previously been reported from several hydrozoan oocytes. Both were found in oocytes of a trachyline medusa by Kessel (1968a) and of *Corydendrium*

parasiticum by Glatzer (1971). Both are also found in the oocytes of hydra. Lipid and glycogen accumulate in interstitial cells which later become incorporated into the hydra oocyte as shrinking cells or pseudocells. Eventually these resemble spherical masses composed largely of glycogen particles and lipid droplets, which may also occur free in the ooplasm (Stagni and Lucchi, 1964). Glycogen and lipid may be the only form of yolk in hydra oocytes. Oocytes of *Spirocodon saltatrix* (Kawaguti and Ogasawara, 1967) and *Sertularella polyzonias* (Honegger, 1980) also contain glycogen but have not been reported to contain any lipid droplets.

Chapter 6, Section G: Nuage Material

RESULTS

Nuage and related material

Nuage material was found in even the earliest oocytes, and has been described in Chapters 4 and 5. In these small oocytes, the nuage occurs principally in two forms, which were termed dense nuage and fibrillar nuage. Both these forms are also found in larger, vitellogenic oocytes (Fig. 6.148). However, a third form is more abundant in larger oocytes, and has been termed particulate nuage. All three forms are usually found in close association (Figs. 6.148-6.150), although areas of fibrillar nuage are sometimes found alone or associated with glycogen (Fig. 6.151).

Particulate nuage consists of aggregations of small dense particles, each some 10 nm in diameter. A single deposit may contain several hundred such particles, and several deposits may be found in a single oocyte section. At high magnification, the particles themselves appear to be composed of a finely granular material (Fig. 6.152). In small, endodermal oocytes, nuage is often associated with other structures, including glycogen, vesicles and areas of dense cytoplasm, but particulate or mixed nuage deposits in vitellogenic oocytes show no such associations. They are often found around the mitochondrial cloud, but very rarely within it (Fig. 6.153). For a period early during vitellogenesis, nuage material is commonly found close to the nuclear envelope, as will be described below. Otherwise, it is distributed apparently at random through the ooplasm.

Nuage appears to be relatively less abundant in oocytes over 100 μm in diameter. This is probably due to an increase in other components rather than a reduction in the quantity of nuage within an oocyte. Dense, fibrillar and particulate nuage are also found in male germ cells (see Chapters 11 and 12), but have not yet been observed in embryos or larvae.

Curious, elongate fibrillar structures are also found during vitellogenesis (Fig. 6.154). They may be 3-4 μm long, and consist of fibrils in a hooped or coiled arrangement. The individual fibrils are some 15 nm in diameter, and may show a very precise patterning (Fig. 6.155). These structures have always been found associated with glycogen deposits, but it is not known whether they are related to the less regular fibrillar structures described in Section F of this chapter. Smaller versions of these structures have also been found in endodermal oocytes and early male germ cells, but not as yet in non-germinal cells. Very occasionally, less precisely arranged elongate fibrillar structures were found in vitellogenic oocytes (Fig. 6.156). These are not associated with glycogen, and have not been found in male germ cells. Their significance is unknown.

On several occasions, other structures which may be related to nuage were found near or among the masses of small vesicles often associated with the trophonema (see Chapter 7). These structures consist of bands of dense material separated by less dense material showing vague transverse striations (Figs. 6.157, 6.158). Some of the bands may be curved or even forked. On one occasion these structures were situated near a pair of centrioles, but again, their significance is unknown.

Association of nuage with the nucleus

For a period early during vitellogenesis, during the early stages of compound yolk granule formation, dense nuage material is very commonly found immediately adjacent to the nuclear envelope. There also seems to be a correlation between the position of the nuage and the nucleolus and nuclear fibrillar bodies. Most commonly, the nucleolus is situated close to the nuclear envelope, and nuclear fibrillar bodies are found between the two. The nuage lies outside the nuclear envelope at this point, and may be precisely localized opposite the fibrillar bodies (Figs. 6.159, 6.160). Sometimes the nuclear envelope is indented in this region, and the fibrillar bodies may be located in depressions in the surface of the nucleolus (Fig. 6.161). Less commonly, the nuclear fibrillar bodies lie further from the nucleolus, and sometimes a band of dense material extends between the two (Fig. 6.162). It seems likely that the nuage and the nuclear fibrillar bodies interact across the nuclear envelope, but the passage of material through the envelope was never observed.

DISCUSSION

Structures known as nuage have been reported from the germ cells of a great many animal species, ranging from coelenterates to mammals (Eddy, 1975; Roosen-Runge, 1977). Nuage is of interest principally because of its possible role in the determination of the germ cells. The idea that some material, usually known as 'germ plasm', is present in germ cells and fertilized eggs and causes cells which receive it during cleavage to later give rise to germ cells, can be traced back a hundred years or more (Nussbaum, 1880; Weismann, 1892). A number of more recent studies have provided evidence that a

cytoplasmic germ cell determinant does operate in some invertebrates and non-mammalian vertebrates (Beams and Kessel, 1974; Eddy, 1975; Mahowald *et al*, 1979). In *Drosophila*, for example, granules present in the cytoplasm of the egg, known as polar granules, become incorporated into a limited number of cells during cleavage and embryogenesis, and the progeny of these cells give rise to the germ cells. If the region of the egg containing the polar granules is destroyed, sterile adults are produced. This effect can be overcome, however, by transplanting normal polar granules from another egg (Illmensee and Mahowald, 1974, 1976; Okada *et al*, 1974). A similar situation appears to exist in anuran amphibians, and comparable experiments have been performed (Smith, 1966; Smith and Williams, 1975; Eddy, 1975).

The cytoplasmic constituents thought to represent the germ plasm have been observed with the electron microscope for both insects (e.g Mahowald, 1962) and amphibians (e.g Balinsky, 1966; Mahowald and Hennen, 1971), and have now been followed throughout the life history of some members of these groups (Eddy, 1975). The ultrastructural appearance of the germ plasm varies between species and during different stages of the life history. However, it generally appears as irregular dense masses of fibrous or fibrogranular material which lack a limiting membrane (Eddy, 1975). Material conforming to this description has now been described from the germ cells of many species from other groups. Since in most cases this material has not been followed throughout the life history of the animal and experimental studies have not been carried out to confirm its function, it is perhpas presumptive to call it germ plasm. The term nuage was first employed by André and Roulller (1957),

and is perhaps more appropriate for these structues, since it is descriptive of their appearance but does not imply a particular function.

In *A. fragacea*, the nuage material exists in several forms and changes in appearance during germ cell development. Both these types of variation are common in other species (Eddy, 1975), and the overall appearance of the nuage is very similar to that of, for example, some amphibians (Kerr and Dixon, 1974). It is perhaps to be expected that the nuage will change in form with time. Nuage may be required to remain in a quiescent form for long periods, and there must also be periods of nuage synthesis if its amount is not to become attenuated during cell divisions. The association of nuage with the nucleus has been observed in many other species, and has usually been taken as evidence of nucleo-cytoplasmic exchange. Several authors have gone further and proposed that nuage is formed from material originally present within the nucleus, and often in the nucleolus (Eddy, 1975). From the present study, there is some evidence that the nuclear fibrillar bodies derive from the nucleolus (see Section A of this chapter) and then interact with the nuage across the nuclear envelope. The form or direction of this interaction is not clear; no material was ever observed in transit through the nuclear envelope. However, it is perfectly possible that such an interaction could be mediated by molecules or particles too small to be visualized by electron microscopy.

Among coelenterates, nuage has been observed and illustrated from spermatogonia of the hydrozoan *Phialidium gregarium* by Roosen-Runge (1977), and seems similar to that of *A. fragacea*. 'Germinal plasm' has been reported from

Pelmatohydra robusta by Noda and Kanai (1977). This material was found in oogonia, interstitial cells and developing cnidocytes, and has more recently been reported from a range of other cell types (Noda and Kanai, 1980) so its significance is unclear. Structures resembling nuage have been described from oocytes of an unidentified trachyline medusa by Kessel (1968a) and from oocytes of *Corydendrium parasiticum* (Glatzer, 1971), *Eudendrium armatum* (Wasserthal, 1973) and *Podocoryne carnea* (Boelsterli, 1977). The last three authors suggested that these structures might be involved in the production of ribosomes or yolk material.

The fact that during the present study, for the first time among coelenterate species, identical nuage material has been found in both male and female germ cells, and not in somatic cells, may be significant. Since the biochemical activities of male and female cells are likely to be very different, it is unlikely that the nuage is involved in any of the synthetic or metabolic processes of these cells, or involved with yolk production. It seems more probable that nuage is indeed involved in the process of germ cell determination. Both Nieuwkoop and Satasurya (1981) and Mahowald and Boswell (1983) consider that the differentiation of germ cells from pluripotent interstitial cells in coelenterates is an epigenetic process, in other words brought about by interaction with external influences rather than conforming to an inbuilt programme within the individual cells themselves. However, even in hydra, there is now some evidence for a separate, sub-population of interstitial cells, pre-destined only for germ cell differentiation (Littlefield, 1984).

The origin of the germ cells in sea anemones is still uncertain, but the presence of nuage, apparently comparable to that of higher organisms, suggests that some degree of segregation of the 'germ cell line' may occur.

Chapter 6, Section H: The Oocyte Surface - Cortical Granules and Cytospines.

RESULTS

1. Cortical Granules

Cortical granules are the last major type of cytoplasmic inclusion to appear during oogenesis. They usually only begin to appear after compound yolk granule synthesis is well under way and fibrillar granule formation has begun to decline, usually at an oocyte diameter of 80-100 µm.

Fig. 6.163 shows a low power view of a peripheral region of a large oocyte containing numerous cortical granules. These are very dense, membrane-bound bodies which are usually spherical or slightly elongate, but which may be more extended or dumb-bell shaped (Fig. 6.164). They average 350 x 500 nm in size. They are always more common at the periphery of the oocyte than in the centre, and their cortical distribution becomes more marked as the oocyte nears maturity. The contents of the granules are very electron dense and at high magnification appear to be finely granular, but otherwise appear homogeneous and show no sub-structure. The limiting membrane is smooth in outline and closely applied to the granule contents (Figs. 6.165, 6.166). The granules may be situated very close to the oocyte plasma membrane.

Formation of cortical granules

Cortical granules appear to be produced by Golgi complexes. In the peripheral cytoplasm of large oocytes, Golgi complexes are often found with small granules with highly electron dense contents. These complexes are rather different in appearance from those found associated with fibrillar

granules (see earlier section), and were not observed in smaller oocytes containing no cortical granules. The complexes consist of stacks of 7-10 flattened cisternae, which are usually curved and in some section planes may appear circular (Fig. 6.167). Sometimes several stacks are grouped together to form larger complexes (Fig. 6.168). The stacks are usually associated with very large numbers of small membrane-bound vesicles, 60-100 nm in diameter, containing a material of moderate electron density. The contents of the cisternae usually increase from the outer or convex face of the stack to the inner, and, in particular, the innermost cisternae may contain swollen regions filled with highly electron dense material (Figs. 6.167, 6.168). These regions usually occur at or near the end of a cisterna, but may also occur near its centre. The inner aspect of the stack may be associated with many small vesicles and two types of larger granule, dense granules and dense-cored granules. The dense granules contain material similar to that in the distended regions of the innermost cisternae (Fig. 6.168), which in turn is similar to the contents of the cortical granules. The dense-cored granules are usually larger, up to 0.5 μm in diameter, and often have a loosely-fitting membrane. Their contents are of slightly lower density than the dense granules, but they always include a single, spherical core of more dense material. The outer material appears coarsely granular, but the cores are finely granular and resemble the contents of the dense granules and cortical granules. They may also have a fuzzy coat some 20 nm wide outside the membrane (Fig. 6.169). Both types of granules show signs of fusion with the small vesicles found around them.

Golgi complexes involved in fibrillar granule formation are usually closely associated with one or two cisternae of rough endoplasmic reticulum (see Section D of this chapter). Some complexes involved in cortical granule production show no obvious association with endoplasmic reticulum (Fig. 6.170). Others may be associated, but in a less precise way (Figs. 6.167, 6.168, 6.171).

Low-density granules

During the later stages of oogenesis, after cortical granules have begun to appear, small numbers of membrane-bound granules of variable appearance are found close to the surface of the oocyte. Their contents are always less dense than those of the cortical granules, and so they have been termed low-density granules. They range in size from 0.2 to 0.8 μm in diameter, and are usually roughly spherical. Their contents vary greatly in appearance. Some contain granular material of moderate electron density (Fig. 6.172), while others contain only a finely flocculent material of very low density (Fig. 6.173). Some may contain very small dense particles. The limiting membrane is often loosely fitting, perhaps more obviously so around the lowest density granules. Very rarely, these granules are observed to fuse with the oocyte membrane and apparently discharge their contents to the outside (Fig. 6.174). Many more granules are seen lying very close to the oocyte membrane (Fig. 6.175), and some may bear a protuberance on the side closest to the oolemma which almost touches it (Fig. 6.176). In other cases, the granule membrane runs parallel to the oolemma, separated from it by a constant and narrow gap of some 20 nm, and following its undulations.

A number of low-density granules also appear to contain a fibrillar component. This may range from a small area showing a fibrillar sub-structure (Figs. 6.177, 6.178) to an obviously fibrillar mass which may occupy a significant proportion of the granule (Figs. 6.179, 6.180). The fibrils appear to be X-shaped in cross-section, and appear identical to those found in fibrillar granules (see Section D of this chapter). In some cases, granules ahich appear to be intermediate between fibrillar granules and low-density granules may be seen (Fig. 6.181).

While low-density granules only occur in the peripheral layers of the oocyte, and many lie close to the oolemma, others are found up to 5 μm away from it. It has not been possible to correlate the appearance of the granule contents with their closeness to the oocyte surface; granules of all appearances may be found immediately adjacent to the membrane, or some distance away from it.

2. Cytospines

The oolemma of all but the smallest oocytes bears microvillus-like cylindrical projections known as cytospines. Cytospines on small oocytes tend to be small and sparsely scattered (see Chapter 5) but during vitellogenesis they become larger and arranged in definite tufts or clumps, usually folded back against the surface of the oocyte. The abundance of the cytospines varies over the surface of the oocyte; very commonly individual tufts are separated by a few microns of free oolemma, as in Fig. 6.182, but in places the cytospines may be so densely packed that individual tufts merge together (Fig. 6.163). Sometimes quite extensive areas can be found free of cytospines (Fig. 6.164).

The cytospines even within a tuft may vary considerably in diameter. Usually those near the centre of the tuft are larger and may be 400 nm in diameter, while some near the outside may be only 150 nm (Fig. 6.183). They appear to remain of constant diameter along most of their length, however. Some cytospines may extend for up to 12 μm in length. Further information about the arrangement of cytospines within tufts was obtained from the examination of spawned eggs, and is discussed in Chapter 13. Usually cytospines within a tuft are closely packed and take up interlocking shapes. They are often separated by a fairly constant intermembrane gap of about 25 nm which may be a result of the interaction of their glycocalyces. Near their points of insertion into the oolemma, the cytospines may be irregularly shaped and are often fused in groups of two or three (Fig. 6.184). Some appear to originate in depressions in the oocyte surface (Fig. 6.185).

The centres of the cytospines contain a fibrillar material. In oocytes less than 50 μm in diameter, this material tends to be poorly defined and indistinct, but in larger oocytes it can be seen to consist of filaments, each about 5 nm in diameter, running longitudianlly along the cytospines, and projecting from their bases as rootlets (Fig. 6.186). The number and arrangement of the filaments varies greatly between different oocytes, possibly reflecting the quality of fixation. In general, the number of fibrils would seem to increase with the size of the oocyte. The cytospines of some large oocytes are almost filled with filaments which may be arranged in a regular lattice when seen in transverse section (Fig. 1.187). Sometimes they are arranged in a hexagonal array, with a centre to centre spacing of some 12 nm, while in

other cases they appear to be aligned in rows (Fig. 6.188); 200-300 filaments may occur in each cytospine. There may be gaps or irregularities in the lattice, however, especially near the cytospine membrane (Fig. 6.189), and the filaments appear to be more regular near the bases of the cytospines. The filaments continue from the bases of the cytospines, forming rootlets which may extend for up to 1.5 μm into the cytoplasm (Figs. 6.190, 6.191). The rootlets are always of smaller diameter than the cytospines, due, at least in part, to a more compact arrangement of the filaments. In the rootlets, the spacing between the filaments may be only 9 nm centre-to-centre. For this reason, rootlets appear more dense than the cores of the cytospines from which they emerge. The rootlets taper along their length, apparently because of a progressive reduction in the number of filaments they contain rather than a change in their arrangement. The rootlets may curve at the base of the cytospines (Fig. 6.192), and since not all the rootlets from a single tuft always curve together, they may appear tangled and confused (Fig. 6.193). Sometimes neighbouring rootlets appear to fuse to form larger, irregular masses of filaments, which may appear to separate later (Fig. 6.194). Otherwise, however, there appears to be no system of transverse filaments linking the rootlets to each other or other cellular components. Cortical and fibrillar granules are occasionally found among the rootlets of a tuft of cytospines, and, if so, the granules are often elongate and aligned along the axis of the rootlets (Figs. 6.195, 6.196). However, this association is not thought to have any functional significance (see Discussion).

Occasionally groups of 10 nm filaments are seen just beneath the oolemma, and usually aligned at an acute angle to it (Fig. 6.197). They appear to terminate just beneath the membrane, and are not apparently associated with the cytospine rootlets or any other organelles.

Whorls of membranous myelin-like material are sometimes found immediately beneath the oolemma (Fig. 6.198). It is possible that these are discharged to the outside, since similar whorls also occur outside the oocyte, often between the bases of the cytospines (Fig. 6.199).

Tufts of cytospines may also be involved with the pinocytotic uptake of materials from the exterior of the oocyte. Coated and uncoated vesicles are often found close to the bases of cytospine tufts, and coated vesicles are often found apparently forming between the insertions of neighbouring cytospines (Fig. 6.200). Less commonly, such vesicles can be seen forming on the oocyte surface generally, not associated with cytospines (Fig. 6.201). These vesicles are usually some 80 nm in diameter, and bear a bristle coat on the cytoplasmic side of the membrane, and a thin layer of finely granular material on the central surface, originally facing the exterior. Otherwise they contain material of very low electron density. Some small vesicles close to the surface of the oocyte appear to enclose smaller, membrane-bound vesicles (Fig. 6.202), but their mode of formation is unclear.

DISCUSSION

Cortical Granules

Cortical granules have been reported from a very wide range of animal oocytes, from coelenterates to mammals (for a review, see Guraya, 1982). In many cases, but not all, they are discharged to the outside of the egg after fertilization as part of the cortical reaction, and so function to help prevent polyspermy. This will be discussed in greater depth in the section on early development (see Chapter 13).

The structure of these cortical granules is often more elaborate than the simple membrane-bound sacs of homogeneous electron dense material found in *A. fragacea*. Among the echinoderms, which have been very extensively studied with regard to cortical granule function (for reviews see Schuel, 1978; Shapiro and Eddy, 1980), the granules of each genus may exhibit a characteristic internal fine structure (Runnström, 1966). Those of *Arbacia* contain an electron dense stellate core, while those of *Strongylocentrotus* exhibit spiral lamellae (Anderson, 1968). From other phyla, those of the mollusc *Mytilus edulis* include a bundle of parallel microtubules (Humphreys, 1967), while those of the polychaete annelid *Nereis* are large and contain a diffuse fibrous material (Fallon and Austin, 1970). All the previously described cortical granules from coelenterate species are simple and resemble those of *A. fragacea*, however. Honegger (1983) illustrates a dense, homogeneous cortical granule from an oocyte of *Hydra carnea*, but mentions cortical granules with heterogeneous contents in the text. Some other hydrozoan oocytes do not appear to contain cortical granules, however,

as they are not mentioned as occurring in *Spirocodon saltatrix* (Kawaguti and Ogasawara, 1967), an unidentified trachyline medusa (Kessel, 1968a),*Aequorea aequorea* (Szollosi, 1970), *Corydendrium parasiticum* (Glatzer, 1971) or *Eudendrium armatum* (Wasserthal, 1973). However, they occur in oocytes of most of the anthozoan species so far examined by electron microscopy. Clark and Dewel (1974) describe cortical granules from oocytes of the sea anemones *Metridium* sp. and *Bunodosoma cavernata*. Those of *Bunodosoma* are homogeneous, membrane-bound, and described as spherical by Dewel and Clark (1974). Schäfer and Schmidt (1980) state that, among the Anthozoa, cortical granules occur only in Palythoa (early Zoantharia), Actiniaria and Ceriantharia. Szmant-Froehlich *et al* (1980), report the formation of a vitelline membrane, consisting of a layer of cortical vesicles just below the cell membrane, in oocytes of the temperate coral *Astrangia danae*. The term vitelline membrane, though now less commonly used than previously, is usually indicative of an extracellular investment, and the significant of their observation is unclear.

In *A. fragacea* oocytes, the cortical granules appear scattered throughout the peripheral ooplasm in oocytes over 80 µm, but become restricted to the superficial 5-10 µm in the fully grown oocyte, and have a definitely cortical distribution in the spawned egg (see Chapter 13). Schäfer (1983) also noted the relatively late appearance of these granules, and their increasingly peripheral distribution during the final stages of oocyte growth. Such a rearrangement during oocyte development has also been reported for echinoderm cortical granules (Runnström, 1966).

The Golgi complex has been implicated in cortical granule formation in many oocytes, including those of echinoderms (Anderson, 1968), molluscs (Anderson, 1969) and annelids (Dhainaut, 1969), and the same would appear to be true of sea anemones. Schafer suggests that cortical granules originate from Golgi complexes which show a marked polarity, with much more osmiophilic material in the distal than the proximal cisternae. This polarity was also observed in *A. fragacea*. In the present study, it appeared that cortical granule synthesis followed the typical rough ER-Golgi pathway. Dilations containing material resembling the contents of the cortical granules formed at any point on the inner Golgi cisternae and small vesicles containing similar contents were found nearby. Cortical granules are apparently formed by the fusion of these vesicles. The dense-cored granules may also represent precursor stages in cortical granule formation. They were only found in the vicinity of Golgi complexes of the type thought to be concerned with cortical granule production, and were never found in large numbers. The material of the dense cores is identical to the contents of the cortical granules, while the remaining contents are more granular and less dense. It may be that the final condensation of the cortical granule precursor material may take place either within the innermost Golgi cisternae, or within the dense-cored granules associated with the Golgi.

Low-density granules

Low-density granules are found in relatively small numbers in most oocytes large enough to contain cortical granules. Many of them appear to be closely associated with the oolemma and, rarely, they were observed apparently discharging their

contents to the outside of the oocyte. It thus seems possible that low-density granules represent stages in the decondensation of cortical or other granule types prior to their discharge. Dewel and Clark (1974) reported the discharge to the outside of a small number of cortical granules prior to egg release in *Bunodosoma cavernata*. They also described a morphological alteration in the appearance of the granules immediately prior to and during discharge. Their contents lose their homogeneous appearance and exhibit a granularity of two different densities. After discharge, the contents become a dispersed flocculent. They suggest that the change in appearance of the granule contents is triggered by contact with the external milieu. Schäfer and Schmidt (1980) convincingly demonstrate the discharge of granules, which they assume to be cortical granules, to the outside of the embryo during early development in *Alfredus lucifugus*.

From the present study, it seems possible that low-density granules are derived from cortical granules, and may be destined for discharge. Cortical granules are the most common granules in the oocyte cortex, and low-density granules do not occur in small oocytes prior to cortical granule formation. However, some findings have emerged which are not wholly consistent with this view. A significant minority of low-density granules contain fibrillar elements which have not been seen in cortical granules, but closely resemble those found in fibrillar granules. Granules which appear to represent intermediate stages in the transformation of fibrillar granules into low-density granules were also often seen. This raises the possibility that at least some of these granules derive from fibrillar rather than cortical granules.

The function served by the discharge of low-density granules, of whatever origin, while the oocyte is still within the gonad is quite unclear. Dewel and Clark (1974) suggested that such a premature discharge might be "accidental", reflecting an inherent labillity or reactivity in the granule and egg plasma membranes. The cortical granules of *A. fragacea* eggs do not appear to discharge as part of a cortical reaction after fertilization as do those of *Bunodosoma cavernata* and *Metridium senile* (Clark and Dewel, 1974), and the whole question of cortical granule function in this species is unresolved (see Chapter 13).

Cytospines

A number of anthozoan eggs and oocytes have been reported to be covered by outgrowths which were known by earlier light microscope workers as spines (e.g Gemmill, 1920; 1921; Spaulding, 1972), and the term has been recently used by Wedi and Dunn (1983). Electron microscope studies by Spaulding (1974) and Dewel and Clark (1974; Clark and Dewel, 1974) showed that the spines consist of bundles of smaller processes. Spaulding called the component structures macrovilli, while Dewel and Clark termed them cytospines. They certainly resemble large versions of the microvilli which are found on eggs of many animal groups (see Anderson, 1974), but most recent authors have preferred the term cytospine. Schmidt and Schäfer indicate that while all anthozoan oocytes bear microvilli, only in some Actiniaria are they large and arranged in tufts and so can be referred to as cytospines. During the present study it was found that *Cereus pedunculatus* oocytes bear only small, randomly distributed microvilli

while the other species studied all have cytospines (see Chapter 10).

Cytospines show a number of features in common with the microvilli which are found in a variety of cell types throughout the animal kingdom. Microvilli from several situations have been the subject of an intense research effort in recent years, and some of these findings will be briefly summarized here. Mammalian and avian intestinal epithelium brush border microvilli have been particularly well studied. They are finger-like extensions of the apical cell membrane some 1 μm in length and 0.1 μm in diameter, and are generally closely packed together. The lumen of each microvillus contains an axial core of 20-40 parallel microfilaments, each about 5 nm in diameter, which have been shown, by reaction with heavy meromyosin and other techniques, to contain actin. The filaments extend into the cytoplasm from the base of the microvillus, and link up with a network of filaments, containing both actin and myosin and known as the terminal web. The arrangements of all these fibrils has been beautifully demonstrated by the quick-freeze deep-etch technique of Heuser and co-workers (e.g Heuser and Kirschner, 1980; Hirokawa and Heuser, 1981; Hirokawa *et al*, 1981). Demembranated brush border microvillar preparations contract rapidly when exposed to Ca^{2+} ions and ATP, (Mooseker, 1976), and although it is not clear whether such contractions occur in intact intestinal cells, the system has been closely studied as a possible example of non-muscle movement. Similar to microvilli are the stereocilia found on hair cells of the cochlea and vestibule of the avian and mammalian ear, and these have also been closely studied. They may reach 3 μm long and be 0.3 μm

in diameter, although many are much smaller. They are often grouped in discrete tufts rather than randomly or closely packed (Hirokawa and Tilney, 1982).

A. fragacea cytospines may reach 12 μm in length and are 0.3-0.4 μm in diameter. Spines as long as 25 μm have been reported from other species (Wedl and Dunn, 1983). Thus cytospines are much larger than brush border microvilli or stereocilia. They also contain an axial core of 5 nm microfilaments, resembling those of microvilli, and Schroeder (1982) showed that these filaments contain actin. However, cytospines may contain 200-300 filaments, and in some cases they are arranged in a regular lattice-like array when seen in cross-section. This does not appear to be the case in microvilli. Cytospine microfilaments extend into the ooplasm from the bases as do those of microvilli, but they do not appear to link up with a terminal web of cross-linking filaments. This finding may be of some significance. In intestinal brush borders, the terminal web links with the desmosomes at the boundaries of the epithelial cells. The apparent absence of a terminal web in *A. fragacea* oocytes may be correlated with the absence of cell boundaries and intercellular junctions. King (1984) recently investigated microvilli from another system lacking cell boundaries, the human syncytial trophoblast, and found that the terminal web was poorly developed. *A. fragacea* cytospines would seem to make excellent subjects for more detailed research into the structure and function of cytoskeletal elements in microvilli, and would provide a useful comparison with the smaller microvilli of vertebrate brush borders.

In *A. fragacea*, while the oocyte is within the mesogloea, the cytospines generally lie folded back against the oocyte surface. After spawning, they erect to form tufts projecting perpendicular to it (see Chapter 13). Spaulding (1974) found that the cytospines of the anemone *Peachia quinquecapitata* could reach 20 μm in length, but while within the gonad the filaments of the core were sparse and rarely showed any orientation. After spawning, however, when the cytospines straighten and become stiff, they appear filled with densely packed, longitudinally orientated filaments. Such a dramatic change was not apparent in *A. fragacea;* the arrangement of the filaments was essentially similar before and after spawning. Apart from their erection at spawning, no evidence of other movement of the cytospines was obtained.

Both cortical and fibrillar granules were found among the rootlets of some cytospine tufts. The filamentous nature of the rootlets led Schäfer and Schmidt (1980) to propose a functional link between the cytospines and the fibrillar granules, which they termed fibrillar, para-crystalline inclusions. They suggested that the granules were produced in the ooplasm and migrated to the periphery of the oocyte where they formed the cytospines. From the evidence of the present study, this seems unlikely. The microfilaments of the cytospines are circular in cross-section and are about 5 nm in diameter. The fibrils of the fibrillar granules are X-shaped in cross-section and about 15 nm across (see Section D of this chapter). It therefore seems unlikely that one directly gives rise to the other. Additional evidence is provided by the finding that *A. equina* oocytes do not appear to produce fibrillar granules, but are covered by cytospines identical

to those of *A. fragilea* (see Chapter 10). The association of different granules with the cytospine bases is not thought to be of functional significance.

Microvilli have been implicated in a range of functions in animal cells, principally concerning absorption but also involving secretion, chemodetection and the storage of membrane material (Alberts *et al*, 1983). They obviously increase the surface area of the cell which may facilitate the exchange of material, and may contain enzymes involved in the breakdown and transport of molecules. It has recently been suggested that the extreme curvature of the plasma membrane produced in microvilli may alter its physical properties and may allow the more rapid passage of some molecules through it (see O'Donnell and Maddrell, 1983). Microvilli are found on the surface of a great many animal eggs and oocytes (Anderson, 1974), and many authors have suggested that they are concerned with the uptake of precursor materials (e.g Anderson, 1974; Eckelbarger, 1983). Schmidt and Schäfer (1980) proposed that the cytospines of anthozoan oocytes were also resorptive organelles. The findings of the present study are also consistent with this view. The cytospines increase in size and number as the oocyte grows and begins vitellogenesis, and may serve to at least offset the fall in surface area to volume ratio as the oocyte enlarges. They may aid the uptake of nutrients over the oocyte surface generally, and they are closely involved with the trophonema, which itself is specialized for the transport of nutrients into the oocyte (see Chapter 7). After spawning, the cytospines may serve a different range of functions, but their formation so early during oogenesis suggests that they are functional while the

oocyte is still within the gonad.

The formation of coated pits and vesicles at the cell surface is thought to be associated with the selective uptake of specific molecules from the exterior of the cell by a process known as receptor mediated endocytosis. In this process, the molecules to be interiorized first bind to receptors on the cell surface. These then migrate to localized areas, which then pinch inwards to form small depressions or pits. Specific proteins, including clathrin, become associated with the cytoplasmic face of the membrane at these points, and may play a cytoskeletal role in curving the membrane to form the pits. The pits then separate from the cell membrane to form coated vesicles (Silverstein and Steinman, 1977; Pearse and Bretscher, 1981; Paston and Willingham, 1981). Receptor mediated endocytosis is very commonly observed in oocytes where yolk component synthesis occurs at a site remote from the oocyte and is carried to the oocyte in the bloodstream as in many insects and vertebrates (Anderson and Telfer, 1970; Wallace, 1978). Endocytosis appears to take place at a moderate level over the entire surface of the *A. fragacea* oocyte. However, a localized region of the oocyte surface adjacent to the trophonema seems to be very much more active in this respect, and will be described in Chapter 7.

Chapter 6, Section 1: Amoebocytes

RESULTS

During vitellogenesis, granular amoebocytes become closely associated with the surface of the growing oocyte. Apart from the trophonema (see Chapter 7), this is the only contact between oocytes and non-germinal cells observed at any stage.

Granular amoebocytes are motile cells of variable appearance which are found in all parts of the anemone, and are common in the mesenteries and gonads. They occur in the mesogloea and among the basal regions of both ectodermal and endodermal epithelial cells. The general features of an amoebocyte are shown in Fig. 6.203. They are often irregular in shape, and so their size in section varies greatly. The nucleus is usually spherical or ovoid, some 3-4 μm in diameter and of only moderate electron density.

The cytoplasm usually contains a small amount of rough endoplasmic reticulum, often arranged around the nucleus (Fig. 6.204). Mitochondria and Golgi complexes are also found, and both have a conventional appearance. A characteristic feature of the cytoplasm is the presence of numerous small electron dense granules. These are usually spherical or slightly elongate, some 300 nm in diameter and up to 500 nm in length. The granules contain a finely granular dense material. Some appear to be homogeneous, but most have an inner region or core of rather different density to the rest of the granule. A small proportion of granules may also contain small vesicles and membranous elements. Basal-body-rootlet complexes are occasionally seen in the cytoplasm,

although flagella have not been observed arising from them. Various heterogeneous membrane-bound inclusions are also found, which are thought to represent phagosomes.

Granular amoebocytes always contain reserve material in the form of glycogen and, to a lesser extent, lipid droplets, but the quantities of each vary greatly from cell to cell. In some cells, glycogen deposits could occupy a very considerable volume of the cell (Fig. 6.204). Very often the glycogen is located at the periphery of the cell, often in lobes containing little else (Figs. 6.205, 6.206). Sometimes the glycogen-filled lobes are only connected to the rest of the cell by a slender neck of cytoplasm (Fig. 6.207). The lipid droplets range in size from 0.4 um to 0.8 um in diameter, and are similar in appearance to those found within oocytes (Fig. 6.205).

Amoebocytes are very often found close to early vitellogenic oocytes (Figs. 6.204, 6.205). Whether they actively migrate towards oocytes soon after entry into the mesogloea, or whether the initial association is merely fortuitous is not clear. Nevertheless, many amoebocytes come to take up a position closely adjacent to oocytes, lying immediately outside the basal lamina (Figs. 6.206, 6.207). The cytospines which protrude from the oocyte surface and extend through the basal lamina may indent the surface of the amoebocytes. Sometimes a depression forms in the surface of the oocyte in which the amoebocyte lies (Fig. 6.208).

As the oocytes enlarge, the amoebocytes usually become flattened around their surface (Fig. 6.209). The mesogloea often thins around growing oocytes, but this does not seem to be responsible for the spreading of the amoebocytes, since

flattened amoebocytes may also be found in areas where the mesogloea remains relatively thick (Fig. 6.210). As the oocytes grow, their covering of cytospines becomes more dense. The amoebocytes tend to lie in the spaces between the tufts of cytospines. The amoebocytes are always separated from the oolemma by the basal lamina, but close contact occurs between them and the cytospine membranes (Figs. 6.209, 6.210). The relationship between an amoebocyte and a large oocyte is summarized in Diagram 10.

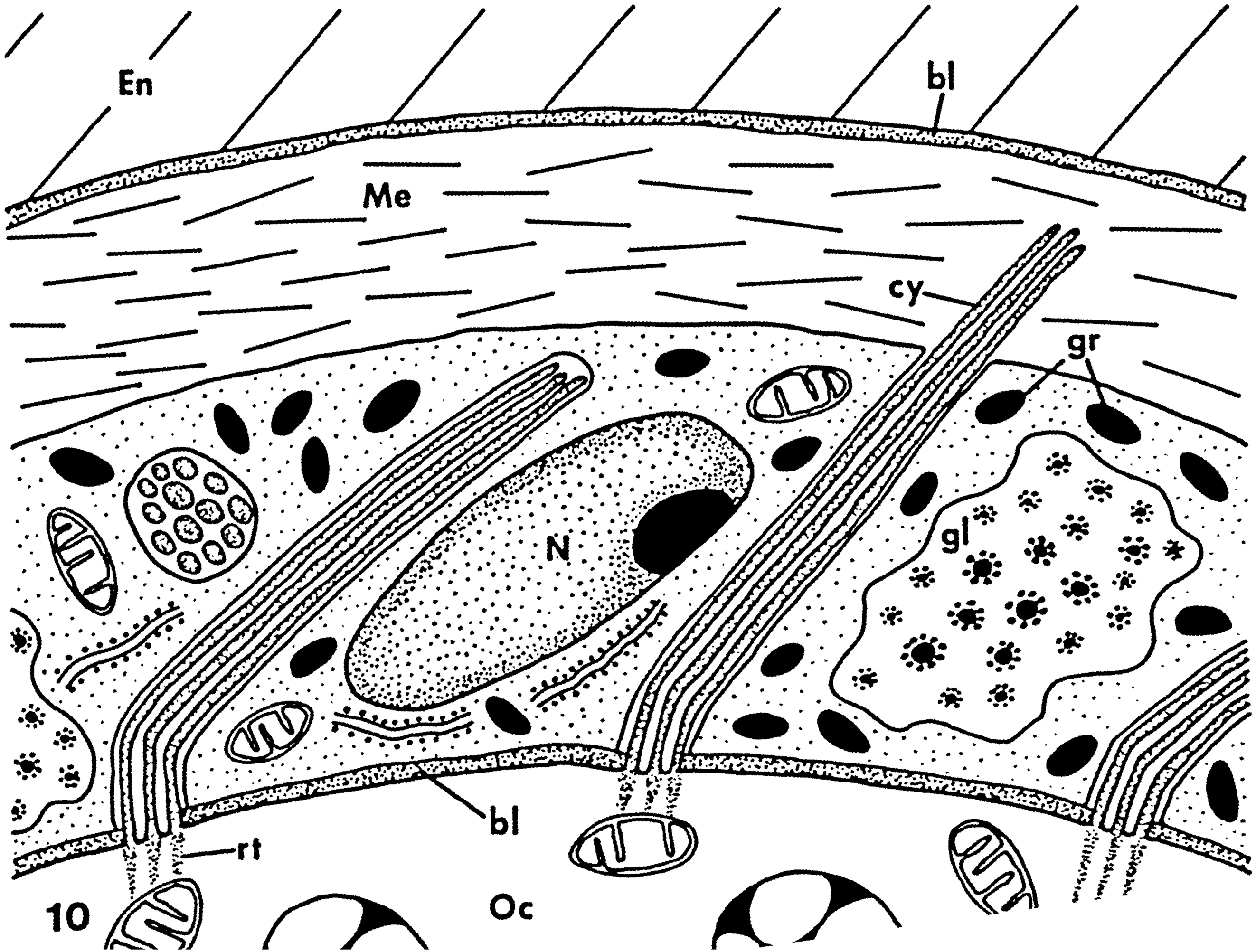
The amoebocytes do not form a continuous layer around the oocyte; large areas may remain free of the association. Amoebocytes associated in this way are very difficult to distinguish with certainty with the light microscope, so it has not been possible to estimate how many amoebocytes may invest an oocyte, or what proportion of its surface they cover.

DISCUSSION

Granular amoebocytes have been mentioned in a number of ultrastructural studies of anthozoan tissues since the pioneering work of Grimstone *et al* (1958). Most authors agree that they are motile, phagocytic cells, and a number of other functions have also been ascribed to them. These include the synthesis of mesogloeal collagen fibres (Bulsson and Franc, 1969; Franc, 1970), the transport of food and waste materials (Van Praet, 1974; Young, 1974) and participation in an inflammatory response (Patterson and Landolt, 1979).
An association between amoebocytes and germ cells has not been previously reported from any coelenterate species. *A. fragacea* amoebocytes are broadly similar in appearance to those

Diagram 10.

Diagram illustrating the relationship between large oocytes and some amoebocytes. Amoebocytes are commonly found flattened around the surface of large oocytes, and lie between the basal lamina and the fibrous mesogloea. Tufts of cytospines may project from the oocyte surface, through the basal lamina and into depressions or channels in the surface of the amoebocyte. The cytoplasm of the amoebocyte contains numerous small dense bodies and extensive deposits of glycogen.


En
bl
Me
cy
gr
N
gl
bl
rt
10
Oc

described recently from the anemones *Anthopleura elegantissima* (Patterson and Landolt, 1979) and *Haliplanella luciae* (Watson and Marlscal, 1983).

Although only a proportion of the oocyte surface is involved, the relationship between amoebocytes and oocytes in *A. fragacea* is so precise and so consistent that it seems unlikely that it could be merely fortuitous. The functional significance of the relationship is not certain, but the amoebocytes may play a role in oocyte nutrition. Most amoebocytes contain considerable quantities of glycogen. Both Boury-Esnault and Doumenc (1979) and Van der Vyver (1981) felt that the storage of glycogen seemed such an important activity for amoebocytes that they should be termed glycocytes. In view of the other functions which they may perform, the less restrictive term amoebocyte is perhaps more suitable, but they may well be important in carbohydrate metabolism. A similar function has been proposed for amoebocytes in other invertebrate groups (e.g Johnston *et al*, 1973).

Amoebocytes or coelomocytes have been implicated in gametogenesis in polychaete annelids by several authors (e.g Dales and Dixon, 1981; Dhainaut *et al*, 1984). In some cases the amoebocytes float freely in the coelomic fluid along with the oocytes, but with no physical contact between them. In spite of their spatial separation, the amoebocytes are important in the lipid metabolism of the oocyte, and may even synthesize yolk protein which is later taken up by the oocyte. The relationship between oocyte and amoebocyte in *A. fragacea* is physically much closer, and it seems quite feasible that they should be involved in the processing and supply of nutrients to the growing oocyte.

Amoebocytes are also found flattened in a corresponding position around testicular cysts (see Chapter 12). They also seem to play an important role in the resorption of degenerate male and female gametes (see Chapters 9 and 12). The association of developing gametes with amoebocytes provides another example, along with the trophonema (see Chapter 7), of germ and somatic cell interaction in a group of animals in which such contact was until recently thought to be minimal or absent (Campbell, 1974b).

SUMMARY

1. In most oocytes, vitellogenesis begins soon after entry into the mesogloea.

2. During early vitellogenesis, the oocyte nucleus enlarges and shows a reduction in overall electron density. There is evidence of nucleo-cytoplasmic interaction involving the nucleolus and nuclear fibrillar bodies during this period, which is not apparent later.

3. A massive band of rough endoplasmic reticulum develops around most of the nucleus during vitellogenesis, and disperses when the oocyte is fully grown. Stacks of annulate lamellae occur among the endoplasmic reticulum.

4. During early vitellogenesis, a large aggregation or cloud of mitochondria forms adjacent to the nucleus. Many of the component mitochondria appear to be highly elongate. The aggregation disperses towards the end of vitellogenesis, and conventional, squat mitochondria are distributed throughout the peripheral ooplasm of the fully grown oocyte.

5. Soon after entry into the mesogloea, the oocyte begins to accumulate fibrillar granules. These are elongate, membrane-bound bodies, 1-2 μm in length, filled with parallel fibrils. The closeness and regularity of the packing of the fibrils shows variation. Most granules are formed in close association with Golgi complexes.

6. Accumulation of compound yolk granules begins later than for fibrillar granules. Compound yolk granules are membrane-bound and roughly spherical, and consist of several

or many spherical, electron lucent inclusions, which resemble lipid droplets, embedded in an electron dense matrix. They appear to be formed by the fusion of small lipid droplets with various types of dense vesicle.

7. Lipid droplets and glycogen are also accumulated during vitellogenesis. During mid-vitellogenesis, very large deposits of glycogen may form, but fully grown oocytes contain numerous small deposits.

8. Several types of distincitve dense structures, lacking a limiting membrane, are found throughout vitellogenesis. Similar structures are also found in early oocytes and male germ cells, and all are thought to represent forms of nuage material.

9. Late during vitellogenesis, cortical granules accumulate in the peripheral ooplasm. They are membrane-bound, slightly elongate, some 500 nm in length and contain a homogeneous dense material. They appear to be Golgi-derived.

10. The oocyte surface becomes covered by distinct tufts of large microvilli known as cytospines. These contain cores of longitudinal microfilaments, which may be arranged in a regular lattice, and which project from the base of the cytospine as a rootlet.

11. Small vesicles are common near the periphery of the oocyte, and there is evidence of pinocytotic activity over the surface of the oocyte generally. The surface close to the trophonema appears particularly active in this respect.

12. Granular amoebocytes become flattened around part of the surface of the oocyte. They lie immediately outside the basal lamina, and make close contact with the cytospines.

13. The central ooplasm of the fully grown oocyte is packed with compound yolk granules, while the peripheral ooplasm contains mitochondria, lipid droplets, fibrillar and cortical granules.

Chapter 7

THE TROPHONEMA

INTRODUCTION

During oogenesis in many sea anemones, a specialized structure develops in a localized region of endodermal epithellium closely adjacent to each oocyte. Such a structure was reported by the Hertwigs during the latter part of the last century (Hertwig and Hertwig, 1879) and was given the name 'Fadenapparat' or filamental apparatus. Nyholm (1943) introduced the term trophonema for this structure, implying that it had a nutritive function. More recently, a number of authors have observed trophonemata associated with oocytes from a range of actinian species (e.g Loseva, 1971a, b; Dunn, 1975, 1982; Dunn *et al* 1980; Jennison, 1979, 1981; Riemann-Zurneck, 1976; Schäfer, 1983; Schmidt and Schäfer, 1980; Wedl and Dunn, 1983). Schäfer (1983) used the older term 'Fadenapparat'. Most of these authors also suggested that the trophonema might be involved in the supply of nutrients to the growing oocyte. However, none of these studies have dealt with the trophonema in any detail and no clear picture of its structure has emerged. Jennison (1979) commented that the development and ultrastructure of the trophonema must be examined further before its function can be determined.

The development of the trophonema begins during the migration of the oocytes into the mesogloea and has been described in Chapter 5. During the following description, the cells which constitute the trophonema are referred to as trophonemal cells. These cells are derived from gonad epithelial cells, and may represent a specialized form of these cells rather than a distinct cell type.

RESULTS

1. Trophonemal Structure During Vitellogenesis

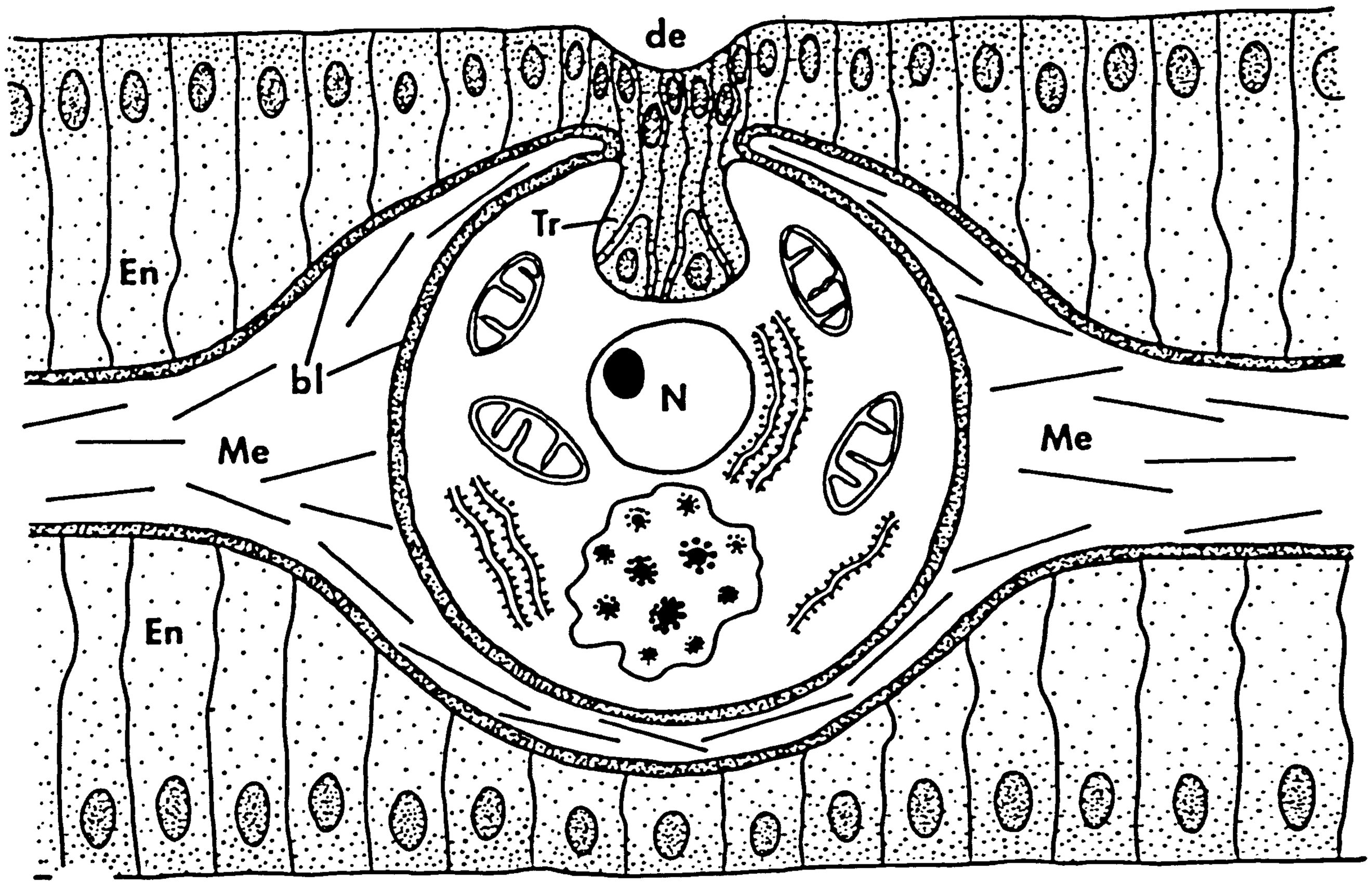
The structure of the trophonema remains similar throughout the period of active oocyte growth and vitellogenesis. Trophonemata can often be recognized under the light microscope (Figs. 7.1, 7.2), but few details of their structure can be distinguished. A much clearer impression can be gained by electron microscopy, however (Figs. 7.3, 7.4). Essentially, the trophonema consists of a gap in the mesogloeal layer surrounding the oocyte, through which the gonad epithelial cells project and make contact with the oolemma in a depression in the surface of the oocyte. The general features of the trophonema during vitellogenesis are shown in Diagram 11.

a) The trophonemal cells

The trophonemal cells originate as gonad epithelial cells, indistinguishable from any others, but they soon undergo specialization. Their apices sink below the level of the surface of the epithelium, producing a depression which can be clearly seen in scanning electron micrographs (Fig. 7.5). The apices of the trophonemal cells are narrower than those of other gonad epithelial cells, and are joined by extensive intercellular junctions of the septate desmosome type. In grazing sections through the trophonemal depression, small channels can be seen running through the intercellular junctions at right angles to the gonad surface (Fig. 7.7). The channels appear as narrow regions where the two membranes are separated, and where no dense material is applied to their cytoplasmic surfaces. The trophonemal cells bear cilia and microvilli which, because of the narrower cell apices, appear more closely packed than on some other areas of the gonad

Diagram 11.

Diagram illustrating the basic structure of the trophonema associated with an oocyte early during vitellogenesis. The trophonema consists of an area in which the gonad epithelial cells project through a pore in the mesogloea and extend into a deep depression in the surface of the oocyte. A depression in the surface of the gonad epithelium also forms at this point, and the epithelial cells involved become specialized. Some may lose their columnar shape, and may have their nuclei located basally, close to the oocyte surface. The basal lamina around the oocyte remains continuous with that of the gonad epithelium.



11

surface (Fig. 7.6, 7.7). The cilia arise in small depressions in the cell surface, and are surrounded by a ring of 9-12 microvilli. They arise from basal-body-rootlet complexes identical to those described in Chapter 3. The apical cytoplasm contains large numbers of closely packed microfilaments, many of which may be associated with the intercellular junctions (Fig. 7.8). Microtubules, some of which are associated with the basal-body-rootlet complexes, are also common. These features combine to make the apices of the trophonemal cells appear much more dense than the surrounding epithelial cells. The trophonemal cell apices contain homogeneous, membrane-bound dense granules and large numbers of small vesicles (Figs. 7.7, 7.8), both of which occur in gonad epithelial cells generally and have been described in Chapter 3.

In the gonad epithelium generally, most of the nuclei are located in the central portions of the cells. In the trophonema, however, the simple epithelial arrangement of the cells becomes obscured, and many nuclei are basally located, near the region of contact with the oocyte (Figs. 7.9-7.11). It is possible that some trophonemal cells do not extend fully from the oocyte surface to the surface of the gonad.

The central and basal regions of the trophonemal cells contain bundles of microfilaments, principally aligned at right angles to the gonad surface (Fig. 7.12). These bundles are more prominent during the later stages of oogenesis, and will be considered in more detail in Section 2 of this chapter. The basal regions contain dense accumulations of glycogen and some lipid droplets (Figs. 7.10, 7.11). Both these reserve materials occur in the gonad epithelium generally

but the concentrations of glycogen in particular appear higher in the trophonemal cells than elsewhere. The trophonemal cells also contain mitochondria, endoplasmic reticulum and Golgi complexes, but not in unusual quantity. Large phagosomes, as described from the gonad epithelium in Chapter 3, were not found in trophonemal cells.

The lip of the pore in the mesogloea through which the trophonemal cells extend may be thickened and appear club-shaped in cross-section. It may also appear indented and irregular in cross-section (Fig. 7.13). Some of the bundles of microfilaments within the trophonemal cells appear to insert on the cell membrane next to the thickened mesogloea. This may be involved with the firm anchorage of these cells to the mesogloeal lip.

b) <u>The interface with the oocyte</u>

In smaller oocytes, perhaps c. 70 μm, the trophonemal depression in the surface of the oocyte tends to be deep and relatively narrow, but becomes more flattened in larger oocytes. At the base of this depression, the trophonemal cell bases contact the oolemma directly. This close contact is interrupted by the tufts of cytospines which project from the oocyte surface, but, between the tufts, the trophonemal cell and oocyte membranes are separated by a very constant gap of about 20 nm. Dense material is deposited on the cytoplasmic faces of both membranes, to produce a form of intercellular junctional complex (Figs. 7.14, 7.15). The complex is not localized into discrete patches, but is found over the entire area of contact between oocyte and epithelial cell membranes. The complex resembles those found between adjacent endodermal muscle processes. No septae have been observed

traversing the intermembrane gap, but these are always difficult to visualize in conventionally prepared anemone tissue. On the trophonemal side of the complex, numerous microfilaments appear to terminate on the membrane (Figs. 7.15, 7.16). On the ooplasmic side, a fine feltwork of fibrils forms a layer some 100 nm thick immediately beneath the membrane (Fig. 7.15).

The interface between oocyte and endoderm may be further complicated by the formation of invaginations into the oocyte into which the trophonemal bases project. These are usually small but occasionally they are extensive and may project a distance of up to 10 μm into the ooplasm (Fig. 7.17). These projections are often filled with glycogen.

While within the gonad, most of the cytospines lie flattened against the surface of the oocyte. In the region of the trophonema, however, they tend to project out from the surface, extending both between the trophonemal cells, and into deep invaginations in their bases. In this way, close membrane contact again occurs between oocyte and endoderm. No junctional complexes or other specializations occur between the membranes concerned, however. The cytospines may indent the epithelial cell bases as large tufts, in smaller groups or even individually. Thus in Fig. 7.18, large groups of cytospines are surrounded by a single trophonemal cell membrane, while in other cases, smaller groups and some single cytospines are enveloped in this way. Fig. 7.19 shows a horizontal section through the mesogloeal pore. In the centre, cytospines projecting directly out from the oocyte surface are cut in transverse section and the trophonemal cell membranes surrounding groups and individual cytospines can be seen.

The surrounding membrane tends to be rather irregular and loose-fitting, instead of following the outline of the cytospines closely. The intermembrane gap varies between 30 and 70 nm.

c) The adjacent ooplasm

The ooplasm situated adjacent to the trophonema is usually highly modified. The oocyte nucleus, which soon after entry into the mesogloea is generally located centrally within the oocyte, becomes displaced towards the trophonema, such that often only a narrow band of ooplasm, 3-8 μm wide, separates the oolemma and the nuclear envelope. During the period of active vitellogenesis, this band of ooplasm usually contains fewer large organelles such as mitochondria, ER and Golgi, and fewer inclusions such as yolk granules or lipid droplets than the rest of the oocyte. However, it usually does contain large numbers of small membrane-bound vesicles of various forms. These vesicles occur elsewhere in the ooplasm, but never in the numbers found beneath the trophonema (Fig. 7.20). The simplest vesicles are roughly spherical, 50-80 nm in diameter, and appear 'empty', or contain a fluid of negligible electron density (Figs. 7.20, 7.21). Others may be larger and less regular in shape, or flattened into tubules or discs. Many enclose smaller membrane-bound vesicles, while a few contain material of low electron density. At least some of these vesicles appear to arise by pinocytosis between the bases of the cytospines. In regions where the cytospines are closely packed, the vesicles may be especially numerous, and the oolemma between them may appear disrupted (Fig. 7.22).

Sometimes very large accumulations of small vesicles may be found, extending around the lateral faces of the nucleus to the sides of the trophonema (Fig. 7.23). Some of these vesicles bear an external bristle coat (Fig. 7.24), and many enclose smaller membranous cores. Some of the cores may in turn contain dense particles. Pairs of centrioles, with associated satellites and radiating microtubules, have been found within these large accumulations of vesicles (Fig. 7.25). Centrioles often occur in the vicinity of the trophonema (Fig. 7.22), but have not been found elsewhere in the oocyte.

When trophonemata are sectioned horizontally, i.e parallel to the gonad surface, it can be seen that the outer surface of the oocyte, outside the edge of the trophonemal depression, may be highly folded (Fig. 7.26). This is in contrast to the rest of the oocyte surface which is generally smooth. The cytospines in this region also tend to be smaller and less precisely arranged into tufts than elsewhere.

2. Trophonemal Structure Late in Oogenesis

The trophonema persists throughout oogenesis, but its appearance changes towards the end of the period of oocyte growth, as vitellogenic activity begins to decline. Many of the micrographs of these late-stage trophonemata are from material subjected to tertiary fixation with uranyl acetate. As a result, the appearance of glycogen in these sections is considerably altered. It can still be recognized as such, however, and some other fine structural details are more clearly visible.

The depression in the surface of the gonad epithelium

is shallower and less distinct than in earlier stages. The apical regions of the trophonemal cells also appear less specialized. The cell apices are wider, so the intercellular junctions are less closely packed (Fig. 7.27). The septate nature of these junctions can just be discerned in this micrograph. The apical cytoplasm still contains microfilaments but these are less numerous and the cells appear less dense than before. Dense granules and small vesicles are perhaps more abundant than previously. Large phagosomes are still absent.

The original epithelial arrangement of the trophonemal cells is now virtually obscured. Many nuclei occur deep in the trophonema, below the level of the mesogloea, and the shape of many of the cells appears highly irregular (Fig. 7.28). It is not possible to determine whether all the cells contact the exterior surface of the epithelium or whether some are entirely contained within the depression in the oocyte. Bundles of microfilaments are even more prominent in later trophonemal cells than before (Fig. 7.29). The cells still contain glycogen deposits, and endoplasmic reticulum and Golgi complexes are present in unchanged quantities.

Towards the end of oocyte growth, the original cup-shaped depression in the oocyte surface becomes wider and more shallow. The pore in the mesogloea does not increase in size proportionally, and becomes much smaller than the depression beneath it (see Diagram 12). Thus the mesogloea may be separated from the oocyte for a considerable area, and is flanked by the gonad epithelium on the outside and trophonemal cells on the inner side (Fig. 7.30). The inner face of the mesogloea in this region bears small, sparsely fibrous

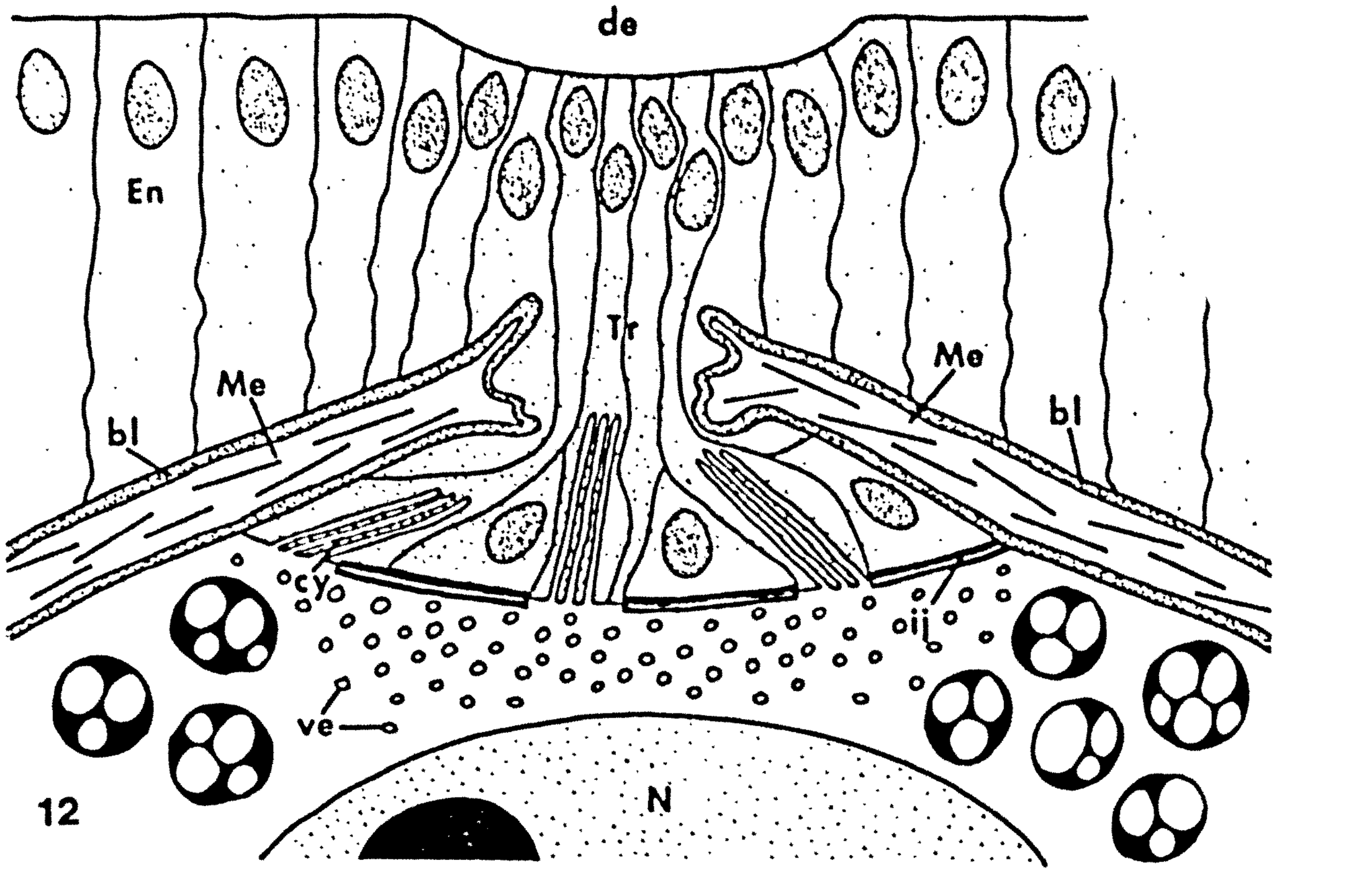


Diagram 12.

Diagram illustrating the structure of a trophonema associated with a large oocyte. Th_ depression in the surface of the oocyte is now wider and flatter, and is considerably wider than the opening in the mesogloea through which the trophonemal cells extend. The lip around the mesogloeal opening is swollen and elaborated, and the depression in the gonad surface is broad and shallow. Tufts of cytospines project among the trophonemal cell bases, and extensive intercellular junctions are formed where these cells contact the oocyte surface.

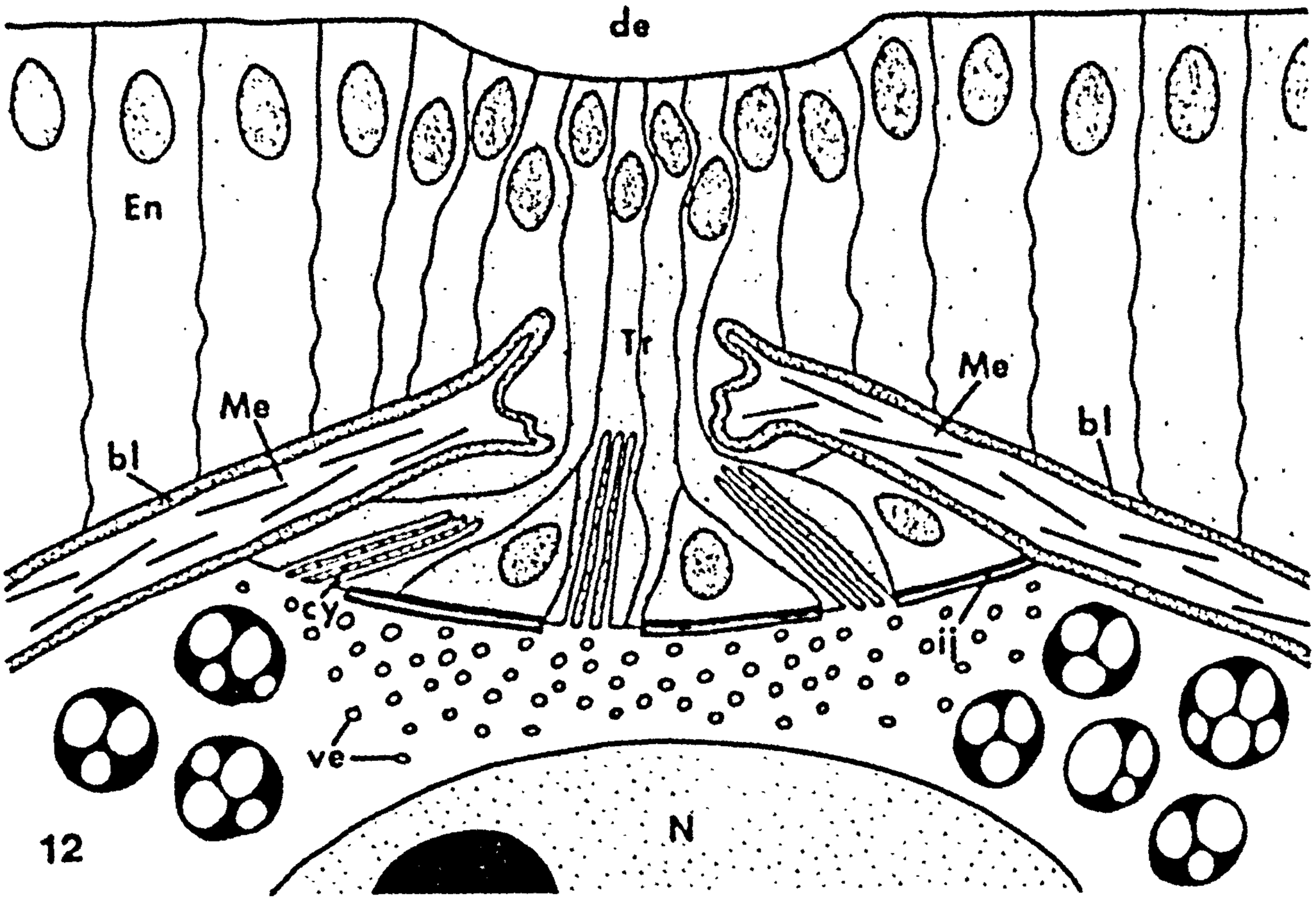


Diagram 12.

Diagram illustrating the structure of a trophonema associated with a large oocyte. Th_ depression in the surface of the oocyte is now wider and flatter, and is considerably wider than the opening in the mesogloea through which the trophonemal cells extend. The lip around the mesogloeal opening is swollen and elaborated, and the depression in the gonad surface is broad and shallow. Tufts of cytospines project among the trophonemal cell bases, and extensive intercellular junctions are formed where these bases contact the oocyte surface.

outgrowths, and well developed muscle processes may lie against it (Fig. 7.31). The muscle fibrils lie parallel to the mesogloeal layer, and are orientated radially outwards from the centre of the pore. It is not clear whether these muscle processes arise from cell bodies located beneath the mesogloea, or whether they originate above the mesogloea and enter through the mesogloeal pore.

The lip of the pore in the mesogloea often shows larger mesogloeal outgrowths than elsewhere. The trophonema cells often appear to curve around the lip, as if pinched by it. Muscle processes may enter through the pore and insert on the mesogloea on the underside of the lip (Fig. 7.32). The mesogloea may also be elaborated at the rim of the trophonema at the point where the mesogloea meets the oocyte. It may bear large, irregular outgrowths of virtually non-fibrous material, against which muscle processes again may insert (Fig. 7.33). Often a tuft of cytospines is found deflected backwards away from the trophonema in this area (Fig. 7.34). Occasionally, outgrowths from the oocyte surface occur in this area. These may be large and irregular, and may form extensive intercellular junctions with the muscle processes (Fig. 7.35).

The relationship between the oocyte and the trophonemal cells remains intimate and essentially unchanged, with close membrane contact and cytospine interdigitation (Fig. 7.36). The cytospines often appear more widely spaced in larger trophonemata. It may be that the number of cytospines or cytospine tufts involved in the trophonema does not increase to keep pace with the lateral expansion of the oocyte depression. The ooplasm immediately beneath the trophonema may still contain small vesicles, but in much smaller numbers than in

earlier stages, and other inclusions, such as cortical and fibrillar granules, are now commonly found in this region (Fig. 7.37).

DISCUSSION

After entry into the mesogloea, the oocytes of *A. fragacea* develop with most of their surface separated from any other cells by two basal laminae and a layer of mesogloea of variable thickness. Amoebocytes may be found closely flattened around parts of the surface of the oocytes (Chapter 6, Section I), but otherwise, the only close contact between the oocyte and other cells is provided by the trophonema. The ultrastructural details obtained during the present study suggest that the trophonema represents a localized and specialized area of intimate contact between the developing oocyte and the endodermal epithelium of the gonad. However, it should not be assumed that the mesogloea and basal laminae surrounding the rest of the oocyte form an effective barrier to transport between oocyte and endoderm. Many classes of molecule may be able to pass through basal laminae and connective tissue spaces and there is evidence of pinocytosis occurring over the oocyte generally (see Chapter 6).

The establishment of the trophonema during the entry of the oocyte into the mesogloea has already been discussed in Chapter 5. Once the oocyte is within the mesogloea, and the last part of it to enter has retracted to leave the cup-shaped depression, specialization of the trophonemal cells begins. It seems possible that the pit in the surface of the gonad epithelium may be formed by the contraction and narrowing of the apical regions of the trophonemal cells. This process may involve the large numbers of microfilaments which occur there,

possibly associated with the prominent apical intercellular junctions. Contraction of cell apices by similar filaments interacting with intercellular junctions is thought to occur in several locations in higher animals, including intestinal brush border epithelia (Mooseker *et al*, 1983). The large number of microfilaments found more basally in the trophonemal cells may also be involved in the maintenance of cell shape and hence the architecture of the trophonema, including the maintenance of the depression in the oocyte surface against its presumable tendency to assume a spherical shape.

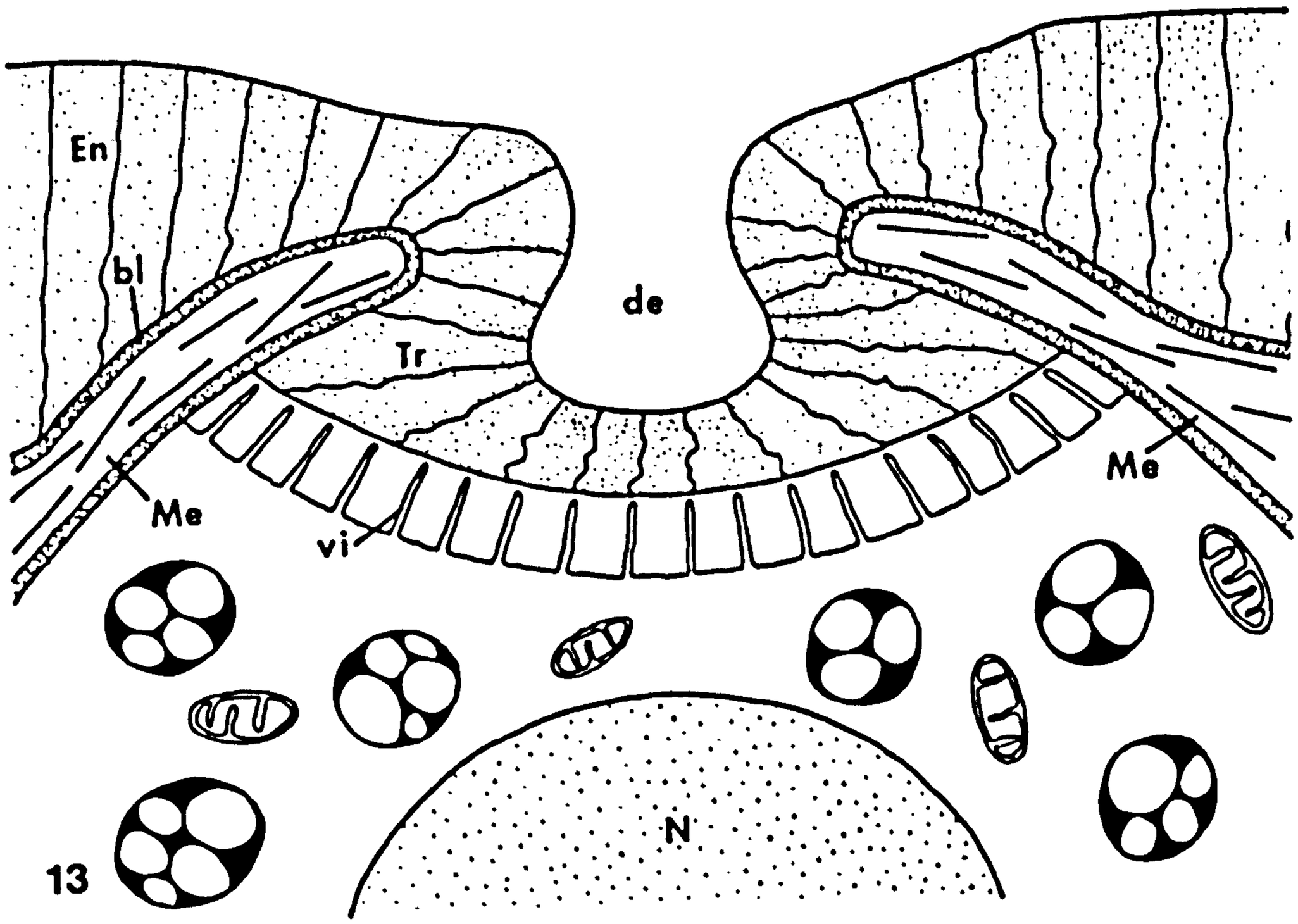
The elaboration of the mesogloea around the lip of the trophonemal pore may originate, at least in part, during the period of oocyte entry into the mesogloea. During the entry of many oocytes, there appears to be an outgrowth of mesogloea around them. This may result in a thickening and extension of the lip region, and an apparent deflection of the lip away from the oocyte after entry is complete. This extension of the lip region may tend to demarcate and partially isolate the epithelial cells in contact with the oocyte, and hence destined to become trophonemal cells, from the rest of the epithelium.

The ultrastructural observations presented here appear to be broadly consistent with earlier light and electron microscope findings, although there appear to be minor variations between different species. Wedl and Dunn (1983) describe the trophonemata of *Urticina lofotensis* as tubular structures, connecting the oocytes, through the mesogloea and the endoderm, to the gastrovascular cavity.

The implication of their description is that the gastrovascular fluid comes into direct contact with the oolemma.

A more detailed examination at the EM level would be required to confirm this suggestion. Schmidt and Schäfer (1980) illustrate a trophonema from *Calliactis parasitica* in which the depression in the gonad surface is much deeper than in *A. fragacea*. The depression extends below the level of the mesogloea, and gives a situation close to that of *Urticina*. A diagrammatic interpretation of their micrograph is given in Diagram 13. In this case, however, a layer of cells separates the oocyte from the gastrovascular fluid. These forms of trophonema were not encountered in any of the species used in the present study.

In *A. fragacea*, the oocyte cytospines appear to be an important component of the trophonema from the earliest stages of its formation (see Chapter 5). Not all sea anemone eggs possess cytospines however. *Cereus pedunculatus* oocytes are covered with smaller microvilli (see Chapter 10), and even these are absent from the region of the oocyte surface adjacent to the trophonema. Some of the major features of the trophonema in this species are shown in Diagram 14. In the *Calliactis* trophonema (Schmidt and Schäfer, 1980; see Diagram 13), the trophonemal cell bases appear to terminate at the level of the tips of the oocyte microvilli. They do not extend between them, and so do not contact the oolemma directly. Schmidt and Schäfer also illustrate a trophonema from *Adamsia palliata* in which a layer of extracellular material, possibly mesogloea, appears to come between the tips of the microvilli and the trophonemal cell bases. In these cases, there would appear to be a very much less intimate relationship between the oocyte surface and the trophonemal cells than is found in *A. fragacea*.



13

Diagram 13.

Diagram showing the apparent structure of the trophonema in *Calliactis parasitica*, as shown in an electron micrograph in Schmidt and Schäfer (1980). The depression in the surface of the gonad epithelium is very deep, and extends below the level of the mesogloea. There does not appear to be close membrane contact between the trophonemal cells and the oocyte surface.

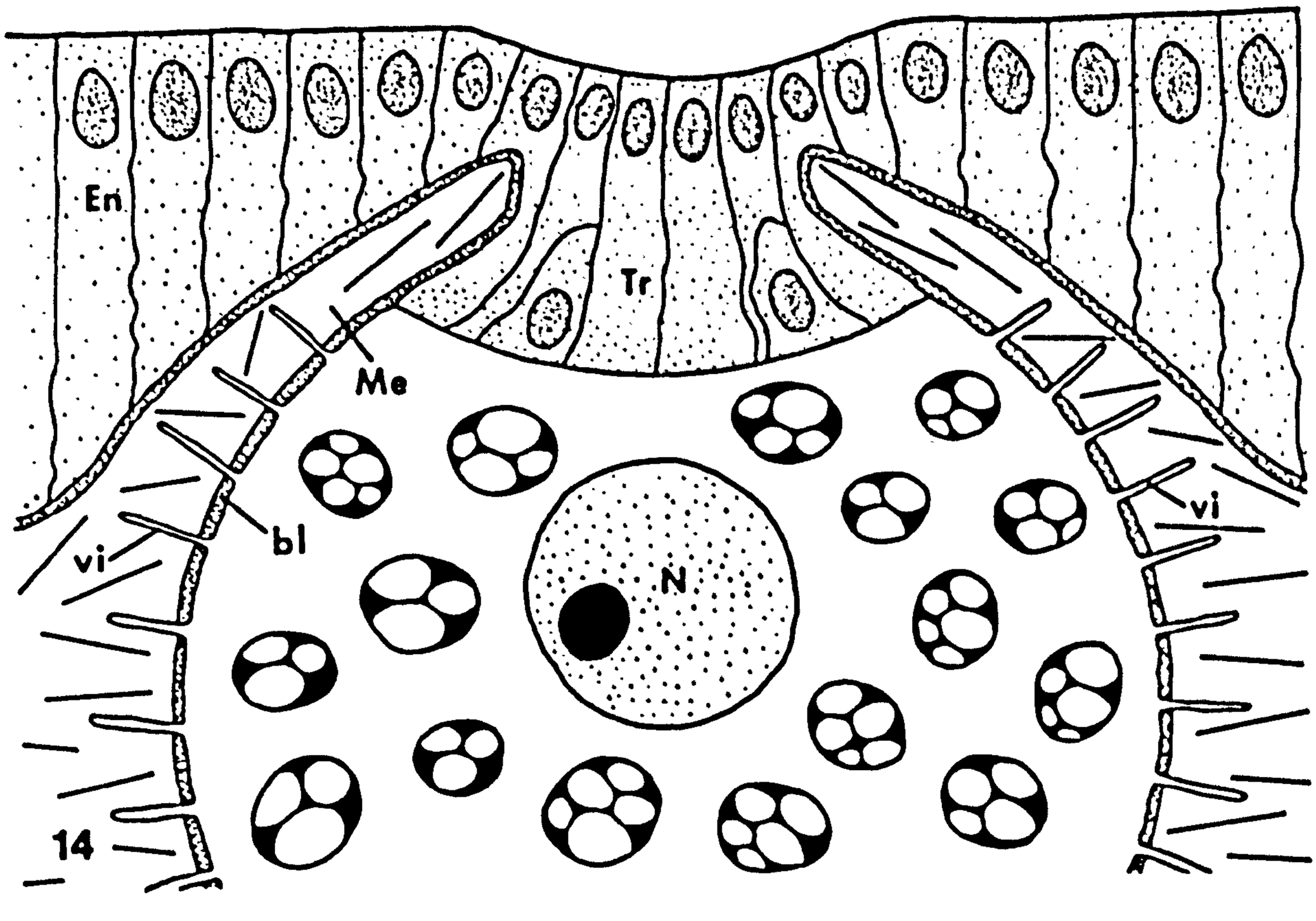


Diagram 14.

Diagram illustrating the structure of the common form of trophonema found in *Cereus pedunculatus*. The oocyte generally bears numerous microvilli, but these are absent from the area of the trophonema. Close contact occurs between the trophonemal cells and the surface of the oocyte, but extensive intercellular junction formation does not take place.

Schäfer (1983) indicates that, in early oocytes, there is an accumulation of mitochondria at the animal pole, where the trophonema is situated. These could provide the energy needed for active transport. In *A. fragacea*, the area of ooplasm adjacent to the trophonema is generally very poorly endowed with mitochondria and other organelles, being largely filled with small vesicles. A mitochondrial cloud is apparent during the early stages of vitellogenesis (see Chapter 6). It is situated next to the oocyte nucleus, and thus could be described as being located at the animal pole, but it has never been observed close to the trophonema. Schmidt and Schäfer (1980) describe the cells of the trophonemata associated with larger oocytes as being vacuolized. The trophonemal cells of *A. fragacea* do not normally appear vacuolized; on the contrary, they often appear more dense and more packed with inclusions than other gonad epithelial cells. In particular, they are usually richly endowed with glycogen. The trophonemal cells shown by Schmidt and Schäfer appear to contain very little glycogen by comparison. Schäfer (1983) mentions the occurrence of yolk-like granules in the trophonemal cells. In *A. fragacea* yolk-like granules were not found in trophonemal cells, in spite of the fact that, at certain times of the year, they may be very common in the gonad epithelial cells generally. It is thought that these endodermal yolk-like granules are derived from the fragmentation of degenerate oocytes (see Chapter 9). Thus they do not represent stages in the synthesis of yolk material for transport into the oocyte.

The overall architecture and the fine structure of the trophonema in *A. fragacea* would appear to be well suited to provide a specialized region for the transport of material

between the gonad epithellum and the developing oocyte. The presence of very large numbers of small, membrane-bound vesicles in the ooplasm immediately adjacent to the trophonema strongly suggests that this region of the ooycte surface is highly active in the uptake of material by pinocytosis. It seems likely that these vesicles are largely formed among the bases of the cytospines. Working on human placental syncytiotrophoblast microvilli, Booth and Vanderpuye (1983) have suggested that protein molecules to be taken up by a microvillar surface may first attach to receptor molecules which are distributed all over the microvillar membrane. The receptors and their bound protein molecules are then swept down to the bases of the microvilli where they are incorporated into pinocytotic pits which then pinch off to become vesicles and enter the cell. The microfilaments of the core of the microvillus may be involved in the basal transport of the receptors. The present findings from the trophonema of *A. fragacea* would seem to be entirely consistent with a similar mechanism operating in this situation. Smaller molecules, such as peptides, amino acids, small sugars and fatty acids may be transported across the membranes of the cytospines; transport systems for all these classes of molecules are known to be present in the membranes of vertebrate intestinal brush-border microvilli (Parsons, 1983). The trophonemal cells contain large quantities of glycogen and significant numbers of lipid droplets; these may be involved in the transfer of glucose and fatty acids into the growing oocyte. The original source of these nutrients is presumably the fluid in the gastrovascular cavity. Trophonemal cells appear to be active in the pinocytotic uptake of material from this fluid,

as is the gonad epithellum generally. Phagosomes, indicative of the uptake of large particles, are however, less common in trophonemal cells than in the rest of the epithellum. No evidence for the uptake of particulate matter, or its transfer to the oocyte, was obtained in the p-esent study.

Thus it would appear that, during vitellogenesis, the trophonema is active in the uptake and transport of small molecules and proteins which are then taken up by the oocyte. This possibility is investigated further, using a more experimental approach, in Chapter 8.

As the oocyte nears maturity, and vitellogenic activity declines, the appearance of the trophonema changes, although it retains all its fundamental characteristics. The ooplasm adjacent to the trophonema comes to contain fewer vesicles but greater numbers of other organelles and inclusions, suggesting a reduction in pinocytotic activity. Within the trophonemal cells, the bundles of microfilaments and the muscle processes become more highly developed, and their insertions on the mesogloea become more elaborate. It seems that the emphasis in trophonemal function shifts from a nutritive role to a mechanical one. As described in Chapter 13, the oocytes are expelled through the trophonema during spawning. It seems possible that the microfilaments and muscle processes of the trophonemal cells may be involved in expelling the oocyte through the pore in the mesogloea, through a corresponding gap which appears between the trophonemal cells and out into the gastrovascular cavity.

A trophonema is also found associated with each testicular cyst in the male gonad (see Chapter 12). These

structures provide interesting examples of somatic cell specialization and involvement in germ cell development, in a group where such associations are not thought to be highly developed.

Chapter 8

OOCYTE NUTRITION

INTRODUCTION

In all coelenterates, the process of digestion takes place in two phases. Ingested food within the gastrovascular cavity is acted upon by a range of enzymes produced by specialized endodermal gland cells. Small particles liberated by this extracellular digestion are then phagocytosed by endodermal cells, and then undergo further digestion intracellularly.

Sea anemones as a group are generally predators, although the range of prey size varies greatly between species.

A. fragacea is capable of ingesting relatively large food items, but little is known of its feeding habits on the shore. Digestion has also not been investigated in this species, but there have been several studies involving the similar and closely related *Actinia equina*. Protease, amylase and lipase enzymes are all thought to be active during extracellular digestion in *A. equina*, and are secreted by cells in the mesenteric filaments (Ishida, 1936; Krigsman and Talbot, 1953). Nicol (1959) reported that the filaments closely surround the ingested food bolus, so the enzymes are released almost directly on to the food and so suffer little dilution by the gastrovascular fluid. Some proteins are hydrolysed at least as far as polypeptides, and the food bolus disintegrates. The intracellular phase of digestion in *A. equina* has been investigated by Van Praet (1976, 1978, 1980). He concluded that specialized cells grouped in parts of the filaments were responsible for phagocytosis and intracellular digestion. Some form of transport system must operate to carry nutrients to the other parts of the anemone, and Van Praet suggested that this might be performed by motile granular amoebocytes.

Thus the mesenteric filaments would appear to play a primary role in both phases of digestion.

The supply of nutrients to the developing germ cells is an important aspect of gametogenesis in all animals. Morphological studies of the gonads in *A. fragacea* (see Chapters 3 and 7) have suggested that the gonad epithelial cells show indications of phagocytic activity, and the trophonema seems well adapted for the transfer of nutrients from the gastrovascular fluid to the germ cells. A series of simple experiments was devised to investigate the role of the gonad epithelium, and the trophonemal cells in particular, in germ cell nutrition. The ability of various endodermal regions to phagocytose food particles was studied using fish blood cells. The uptake of protein molecules by the gonad epithelium was followed using horseradish peroxidase, and the uptake of small molecules by autoradiography. Details of the experimental procedures are given in Chapter 2.

RESULTS

1. Uptake of Fish Red Blood Cells (FRBC)

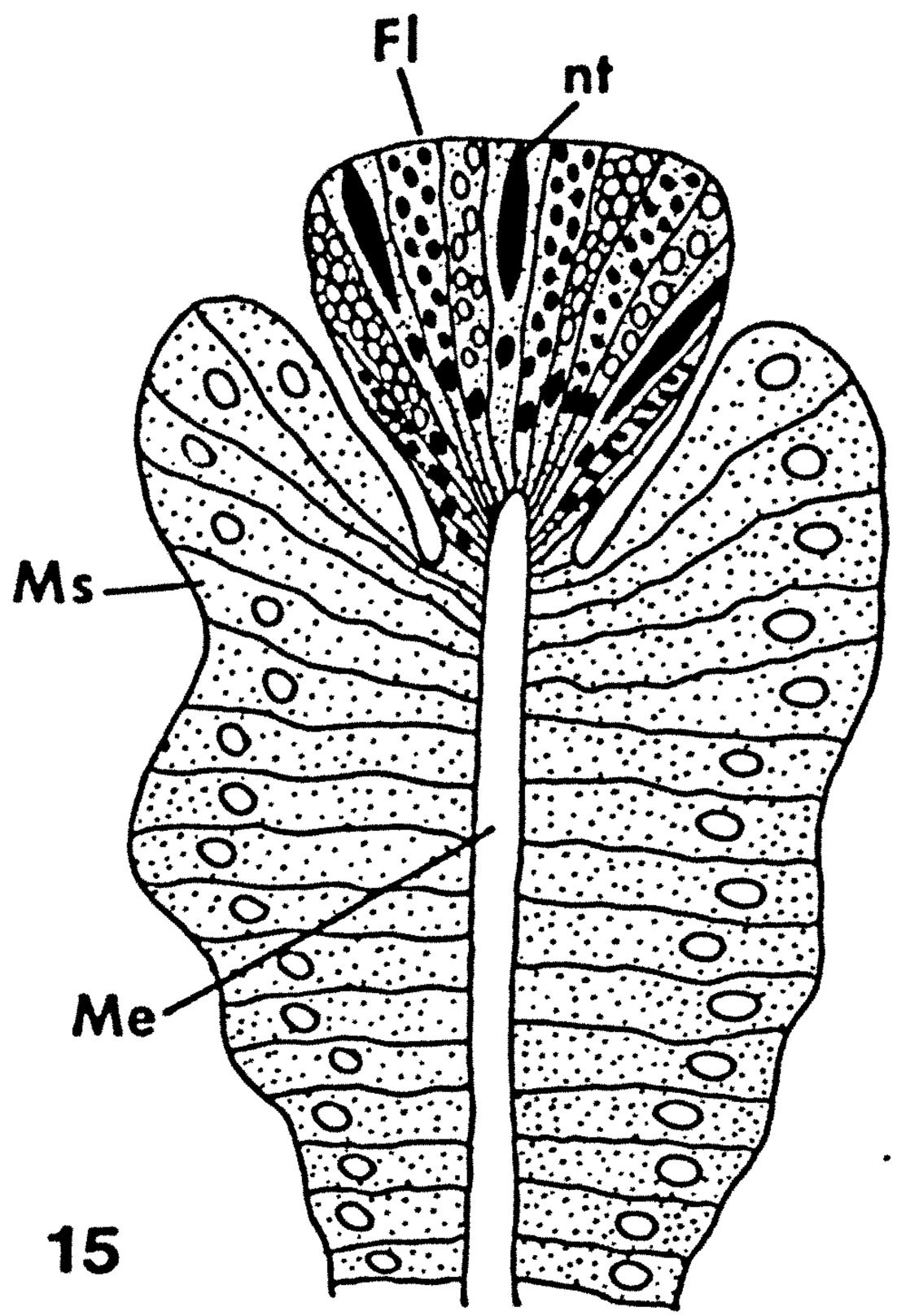
The behaviour of all the anemones after feeding the clots of fish blood was broadly similar. All the clots were readily ingested. Within a few minutes after feeding, all the anemones extended fully and remained extended for several hours if left undisturbed. Those dissected 2 and 6 h post feeding were still extended when taken for dissection. Those which were left for 24 h remained extended for at least 8 h; when observed intermittantly between 8 and 24 h they showed variable degrees of contraction.

In those anemones dissected 2 h post feeding, the lower portions of the mesenteric filaments were found closely applied to the remains of the blood clots in a bolus at the base of the pharynx; the gastrovascular fluid showed a slight pink colouration. By 6 h post feeding, all traces of the ingested clots had disappeared and the filaments had returned to their normal relaxed positions. The gastrovascular fluid was distinctly red in colour and slightly turbid. At 24 h post feeding, the filaments were again in their normal positions, and the gastrovascular fluid was clear and colourless. At no stage during the experiment was any colouration or turbidity of the seawater around any of the anemones observed.

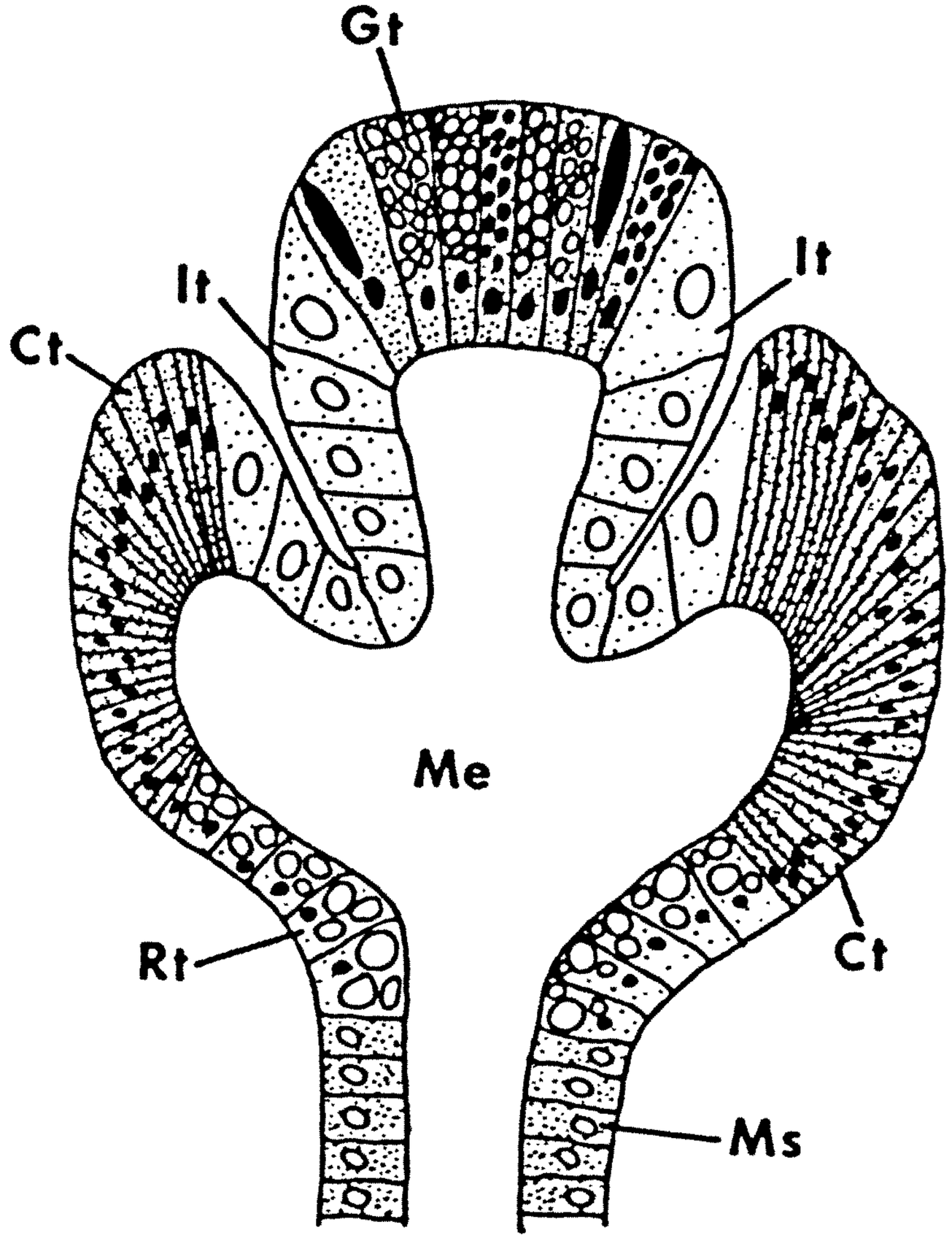
FRBC within endodermal cells could be distinguished clearly in histological preparations. In the anemones dissected 2 h post feeding, moderate numbers of FRBC could be seen in both lower and upper portions of the filaments. The structure of the filaments is shown in Diagram 15. In the lower filament, the thickened region of mesentery immediately adjacent to the filament proper is active in phagocytosis, while the filament itself is not. This localized region of mesentery appears to be functionally linked to the filament, and, during the following description the term lower filament is taken to include this region. Occasional red cells were found in the tentacle and body wall, but none in the general mesentery or gonad endoderm. 6 h post feeding, the endoderm of all regions examined contained FRBC. The endoderm of the tentacles and body wall was densely and evenly packed with large numbers of FRBC (Figs. 8.1, 8.2). The mesenteries

Diagram 15.

Diagram showing the structure of upper and lower mesenteric filaments when seen in transverse section. The lower filament (left) consists of a single lobe containing gland cells and nematocysts. The region of mesentery adjacent to the filament is greatly thickened. The upper filament (right) is trilobed, and is composed of several tracts, differing in cellular composition. The apical cnidoglandular tract contains gland cells and nematocysts, and resembles the lower filament. It is flanked on either side by intermediate tracts, consisting of relatively unspecialized epithelial cells, and ciliary tracts consisting of very slender ciliated cells. These in turn are flanked by the reticular tracts, whose cells contain large, virtually empty vacuoles. The expanded mesogloea of the upper filament contains numerous small, rounded basophilic cells, which for clarity have not been shown in this diagram.



15



contained a lower density, which showed some variation between areas. The expanded mesentery adjacent to the lower filaments contained many FRBC (Figs. 8.3, 8.4). The cnidoglandular and ciliary tracts of the upper filaments contained none, but the intermediate tracts were densely packed and the reticular tracts rather less so (Figs. 8.5, 8.6). The gonads showed a moderate uptake of FRBC, which were distributed patchily rather than evenly (Figs. 8.7, 8.8). 24 h after feeding, the pattern of ingested FRBC was the same as after 6 h, but the overall numbers of red cells which could be recognized clearly in endodermal cells was lower, possibly as a result of intracellular digestion.

Gonad and upper filament material from anemones dissected 6 h post feeding was also examined by electron microscopy. Again, the FRBC could readily be identified in sections. The localized areas of gonad epithelium which contained numbers of phagocytosed FRBC showed occasional pseudopodial extensions from the cell apices, but otherwise were similar to the rest of the gonad (Fig. 8.9). Most FRBC were found singly within closely fitting membranous vacuoles. Occasionally two red cells were found in a single vacuole (Fig. 8.10), and sometimes other material, of unknown origin, was also found within the vacuoles. No FRBC was found in male or female trophonemal cells. This suggests that these cells are not especially active in the phagocytic uptake of food particles, but given the patchy distribution of FRBC in the gonad generally, it does not rule out their involvement altogether.

The EM examination of upper filament material confirmed the light microscope findings. FRBC were absent from the cnidoglandular and ciliary tracts, but present in great

numbers in intermediate tracts, and in lesser numbers in the reticular tracts. The cells of the intermediate tract bear numerous, irregular pseudopodial processes (Fig. 8.11), similar to those mentioned above from the gonad, but larger and more numerous. Intermediate tract cells also contained large heterogeneous inclusions (Fig. 8.12) which may represent residual bodies from previous intracellular digestive activity. The apical regions of the intermediate tract cells contain small dense granules, similar to those found in the gonad epithelial cells (see Chapter 3).

The cells of the intermediate tract are morphologically similar to the gonad epithelial cells. Both are capable of the phagocytic uptake of food particles from the gastrovascular fluid. The intermediate tract cells seem more active in this respect, and show features such as an increased number of pseudopodia which may be related to this. Some FRBC in intermediate tract cells showed a ruffling of their surface (Fig. 8.12), 6 h after feeding. This might indicate the onset of intracellular digestion of the red cells, which was not observed in gonad epithelial cells at this time.

2. Uptake of Horseradish Peroxidase (HRP)

In the first HRP uptake experiment, isolated pieces of gonad were maintained *in vitro* and exposed to HRP in seawater for 30 min or 3 h. Peroxidase activity was then demonstrated by incubating the intact, fixed gonad pieces in Hanker-Yates reagent and hydrogen peroxide. When examined with the electron microscope, the gonad pieces showed reaction products only in the apical regions of the epithelial cells. To check that the absence of reaction product from the central and basal regions

of the cells was not due to poor penetration of the Hanker-Yates reagent, the experiment was repeated using a slightly different procedure. Gonad pieces were exposed to HRP for 2 h and fixed as before. However, prior to incubation in Hanker-Yates reagent, they were cut into 50 µm slices using a freezing microtome. The distribution of reaction product was not altered by this change in procedure. The results from the different HRP exposure times, ranging between 0.5 and 3 h were also identical. The following description therefore does not distinguish between the different treatments.

The apical regions of untreated gonad epithelial cells normally contain an array of small vesicles of various shapes and sizes. Many of these vesicles bear a distinctive internal bristle coat; a small proportion may also have an external bristle coat (Fig. 8.13; see also Chapter 3). Electron dense reaction product, indicative of HRP activity, was found in the apices of all the gonad epithelial cells, although the amount showed some regional variation. The reaction product was localized in membrane-bound inclusions of three main types (Figs. 8.14, 8.15).

i) Dense deposits were found in small vesicles located close to the apical surface of the cell.

ii) Very dense deposits were found in larger vesicles located further away from the cell surface.

iii) Rather less dense deposits were found in elongate vesicles some distance from the surface. These vesicles appear to form part of an irregular tubular system in contrast to the discrete, spherical vesicles described above.

Sometimes reaction product was found deposited on the external surface of the apical plasma membrane, usually in pits or depressions (Fig. 8.16). Similar deposits were also found at the insertions of the microvilli which form collars around the bases of cilia (Fig. 8.17). None of the vesicles containing reaction product showed the distinctive internal bristle coat described above. It may be that the coat is obscured by the dense material.

Trophonemal cells contained variable amounts of reaction product, which was distributed in the same way as described above for other gonad epithelial cells. No reaction product was found in the sub-nuclear or basal regions of any of the epithelial cells, or in germ cells or granular amoebocytes. Control sections of material either not exposed to HRP or incubated in medium lacking hydrogen peroxide also showed no reaction product.

3. Demonstration of Acid Phosphatase Activity

All the gonad epithelial cells examined showed membrane-bound vesicles containing dense, particulate reaction product indicative of acid phosphatase activity. Most of these vesicles were located in the apical regions of the cells, although a small number were found in the basal regions. Most were irregular in shape and between 300 and 800 nm in diameter. Some showed a heavy deposit of reaction product throughout the vesicle (Fig. 8.18, large arrows), while in others the reaction product was less abundant, or restricted to only a part of the vesicle (Fig. 8.18, small arrows). Some heavily labelled vesicles contained characteristic angular electron-lucent inclusions (Fig. 8.19), and are thought to correspond

to the residual body-like inclusions mentioned by Chapman (1974) and others. All of these vesicles appear to represent various forms of secondary lysosome. No smaller, homogeneous labelled bodies which might represent primary lysosomes were found. The dense granules found at the cell apices showed no activity, and neither did the small apical vesicles and tubules discussed in the previous section on HRP uptake (Fig. 8.18). Trophonemal cells showed a similar quantity and distribution of reaction product as the rest of the epithelium.

No reaction product was found in control sections incubated in the absence of substrate or in the presence of sodium fluoride. Some experimental sections showed small dense particles adhering to the apical plasma membranes (Fig. 8.18). These particles were not present in control sections, but their significance is unclear.

4. Uptake of Radiolabelled Precursors

Isolated gonad pieces maintained *in vitro* showed the ability to take up tritiated D-glucose and the tritiated amino acid L-leucine from the external medium. After processing for autoradiography, sections from incubated gonad pieces generated substantial numbers of silver grains after 4-6 days of auto-radiographic exposure. The pattern of silver grain distribution was similar in all treatments for both precursor molecule types. Different incubation and cold chase times made only minor differences. In general, the longer incubation times produced a more intense labelling, while longer cold chase periods reduced the intensity of the labelling.

In all cases, the gonad epithelia and the germ cells were relatively heavily labelled, while the mesogloea was always more lightly labelled. After incubation with leucine,

oocyte nuclei and nucleoli were heavily labelled (Figs. 8.20, 8.21), while after glucose incubation the nucleoli were lightly labelled.

Perhaps the most interesting finding, however, was that the trophonemata were always more heavily labelled than the rest of the gonadal epithellium (Figs. 8.20-8.23). This was true for male and female gonads, for glucose and leucine, and for all incubation and chase times. It suggests that the trophonemal cells are considerably more active in the uptake and incorporation of precursor molecules than other gonad epithelial cells.

In an attempt to distinguish between uptake and incorporation, some gonads were incubated briefly (5 min) in tritiated leucine, then fixed immediately in either formaldehyde or glutaraldehyde. However, the pattern of silver grains produced was the same in each case, and similar to that produced after longer incubation periods.

In all autoradiographs, the background grain density was very low. Unincubated control slides showed very few grains, as did regions of emulsion not overlying gonad material in experimental slides. Control slides also indicated that no chemographic induction or inhibition of grain formation was taking place.

DISCUSSION

The use of blood clots in studies of anthozoan digestion was pioneered by Tiffon and Daireaux (1974). They fed clots of chicken blood to *Cerianthus lloydi* individuals, and concluded that the clots were broken down into individual cells during extracellular digestion, and the

blood cells were then phagocytosed intact by endodermal cells. Clots of fish blood were preferred for the present study, since it was hoped that they would retain their shape better in seawater and so be more easily recognized in sections. The present results seem consistent with those of Tiffon and Dalreaux; the extracellular phase of digestion liberates individual, apparently undamaged cells from the clot, which are then taken up by endodermal cells.

Van Praet (1976, 1980) investigated the endodermal uptake of algal cells in *Actinia equina*. He demonstrated that they were phagocytosed by cells of the intermediate and reticular tracts of the upper filaments, and of the mesentery close to the lower filaments. He also showed acid phosphatase activity in these same cells, and concluded that these represented the sites of intracellular digestion. The products of intracellular digestion must then be distributed to the other regions of the anemone, perhaps by the motile granular amoebocytes. The present study has confirmed that the upper and lower filaments are indeed important sites of phagocytosis and digestion, but has shown that other regions are also involved. All the parts of the anemone investigated showed some uptake, and regions such as tentacle endoderm appeared to be as active as the filaments. Van Praet did not report any activity in other regions, possibly because he confined his histological analysis to the filaments and mesenteries. If, as the present findings suggest, intracellular digestion occurs generally throughout the endoderm, no large-scale transport system for the products of this digestion need be postulated. Some short-range transfer of nutrients is very likely, however, and transfer from endoderm to ectoderm may

still be required. Long range transport appears to be performed by the circulation of the gastrovascular fluid before intracellular digestion begins.

The present study indicates that the gonad epithelial cells are also active in phagocytosis and intracellular digestion. The trophonemata appear to be less active in these respects than the rest of the gonad. This finding is perhaps consistent with the ultrastructural appearance of the trophonemal cells (see Chapter 7). Their apices are narrow and packed with microfilaments, they do not display pseudopod formation, and large phagosomes were not found in their cytoplasm.

Schlichter (1978) reports the occurrence of 'pre-oral' digestion in some sea anemones, including *Actinia equina*. The prey is apparently held at the base of the tentacles, and the anemone partially closes around it. It is digested, before it enters the mouth, by enzymes secreted by both endoderm and ectoderm. This pre-oral digestion liberates dissolved organic material which is then taken up by the ectoderm. No direct evidence for such a mechanism is given, however. Nothing equivalent to this phenomenon was observed either during the present feeding experiments or during the routine feeding of large numbers of *A. fragacea* and *A. equina* maintained in the laboratory. On occasions, anemones in poor condition may remain partially closed during feeding, and may be slow in transferring food to the mouth, but this is not thought to be part of the normal digestive process.

Horseradish peroxidase is an enzyme, with a molecular weight of some 40,000 daltons (Keilin and Hartree, 1951) which has been used as a protein tracer in numerous studies since

its introduction at the electron microscope level by Graham and Karnovsky (1966). It has the great advantage that, because of its enzymic activity, very small quantities can be detected. Most animal cells do not contain significant endogenous peroxidase activity (Straus, 1969). For the present study, the technique of Graham and Karnovsky was modified according to Hanker *et al* (1977), by the replacement of diaminobenzidine in the incubation medium by a mixture of pyrocatechol and *para*-phenylenediamine dihydrochloride, known as Hanker-Yates reagent. Hanker-Yates reagent is thought to be safer in use, more sensitive and more specific than diaminobenzidine in the demonstration of HRP activity.

All the gonad epithelial cells, including the trophonemal cells, were capable of taking up HRP, and presumably other proteins, from the external medium. The HRP apparently binds initially to the plasma membrane, and is then taken up into small vesicles. These vesicles are thought to be the same as those seen to have an internal bristle coat in unincubated sections. Similar, but larger, vesicles have been described from hydra digestive cells (Slautterback, 1967). The HRP is also found in a tubular system & in larger vacuoles deeper within the cell. Since the amount and distribution of the reaction product was the same in gonads exposed to HRP for 3 h as in those exposed for 0.5 h, it seems likely that the apical vesicle and tubule systems are in a steady state, with HRP leaving the system, presumably by degradation, as fast as it is taken up at the cell surface. The presence of numerous acid phosphatase positive vesicles beneath the apical cytoplasm suggests that this degradation may be mediated by lysosomes.

The results of the HRP uptake experiments are entirely consistent with modern theories of receptor-mediated endocytosis. According to these theories, proteins in the external medium bind to receptors situated on the apical plasma membrane which then pinches inwards to form initially a pit and later a cytoplasmic vesicle. Ultimately, the contents of the vesicles are degraded by enzymes of lysosomal origin. Recent interest has centred on the problem of whether or how the receptors escape degradation in this way, which would appear to be a wasteful process. It now appears that there is another intracellular compartment which intervenes between the endocytotic vesicles and the lysosomes. This compartment is thought to consist of membrane-bound vesicles or tubules, known variously as endosomes (Helenius *et al*, 1983) transfer tubules (De Bruyn *et al*, 1984) or 'CURL', the compartment for uncoupling receptor and ligand (Geuze *et al*, 1984). Within this compartment, the ligands are detached from the receptor molecules, which are then recycled, possibly via the Golgi apparatus, to the cell membrane. The ligand molecules are transferred to lysosomes and degraded. In *A. fragacea*, the internal bristle coat of many vesicles may be composed of receptors, and the irregular tubular system may correspond to the CURL or transfer tubules. In many mammalian cells, the receptors are highly specific, but in *A. fragacea* gonad epithelial cells they are presumably less selective. Extracellular digestion may produce a whole range of proteins and polypeptides, and the function of the receptors in this case may not be to mediate selective uptake, but simply to concentrate molecules at the cell surface prior to endocytosis.

The autoradiography experiments indicate that isolated gonad pieces can take up and incorporate small molecules from the external medium. Experiments of this sort generally require some care in interpretation. The principle underlying such precursor uptake studies is that autoradiography reveals the location of the radionuclides still present in the tissue after histological processing. As a rule, the original precursor molecules and any small molecules derived from them are not retained by standard fixative procedures and are lost during processing. Only radiolabelled material which has been both taken up by the tissue and incorporated into larger molecules will be retained and visualized by autoradiography. Thus only the distribution of newly synthesized macromolecules is revealed, which may give little information about routes of precursor uptake. A possible complication is that glutaraldehyde fixation may retain a proportion of unincorporated amino acids. During the present study, a comparison was made between glutaraldehyde and formaldehyde fixation after incubation in leucine. The pattern of silver grains obtained was essentially the same in both cases, so it appears that unincorporated amino acid does not make a significant contribution to the overall level of radioactivity under the conditions used here.

Since developing oocytes and male germ cells were found to contain radiolabelled molecules, but were not in direct contact with the external medium, it follows that molecules taken up by somatic cells must have been transferred to the germ cells. The mechanism of this transfer is not clear, but it is interesting that trophonemal cells appear much more

active in the uptake and incorporation of precursor molecules than the rest of the gonad epithellum. Given the intimate contact between trophonemal cells and germ cells (see Chapters 7 and 12) it seems very likely that they are involved in the transfer process. The autoradiography experiments were carried out during the spring, when both male and female germ cells were actively growing. It would be interesting to repeat the experiments in early summer, when vitellogenesis and growth are in decline, to see if trophonemal activity is also reduced.

The present autoradiographic findings are not consitent with earlier reports that in sea anemones only the ectoderm is capable of taking up dissolved organic material. Schlichter (1975) immersed anemones for short periods in seawater containing labelled amino acids, and found uptake only by the ectoderm. She suggested that this might be related to the absence of apical microvilli on endodermal cells. Van Praet (1980) found that when intact anemones were immersed in labelled amino acids for 2 h, labelling was only ectodermal, but after 6 h immersion the endoderm was also labelled. He concluded that a slow transfer from ectoderm to endoderm occurred. Singer (1971) immersed whole and amputated anemones in tritiated thymidine. He found incorporation only by ectodermal cells, and concluded that mitosis did not occur in the endoderm which must therefore be maintained by the immigration of ectodermal cells.

At least a partial explanation of this discrepancy may be that the gastrovascular fluid is not continually exchanged with the external medium, such that endodermal tissues may not

always be exposed to the radiolabelled molecules. It has been assumed that the siphonoglyphs maintain a constant flow of water through the gastrovascular cavity (Stephenson, 1928). However, during the present study, after anemones had been fed fish blood clots, no leakage of red cells to the outside could be detected. This suggests that, at least during extracellular digestion, the gastrovascular cavity can be sealed off.

Further evidence that this is the case is provided by the work of Minasian (1980). In contrast to Singer (1971), described above, Minasian not only immersed anemones in tritiated thymidine, but also irrigated their gastrovascular cavities by means of a pipette inserted through the mouth. Under these conditions, he found uptake by endoderm as well as ectoderm. The findings of Van Praet (1980), mentioned above, could also possibly be explained by the mouth of the anemone remaining closed throughout a brief immersion period, but opening at some stage during a prolonged immersion and permitting uptake by the endoderm.

If the mouth or pharynx can be closed to prevent mixing of the gastrovascular fluid with the external medium, then this fluid can act as an effective circulation system for the distribution of nutrients. Based on the present study and a re-evaluation of some previous work, the following sequence of events during sea anemone digestion can be proposed:

1) When the food bolus reaches the gastrovascular cavity, the filaments press closely against it and release enzymes for extracellular digestion. High local concentrations of these enzymes may be achieved around the food.

2) As the food mass disintegrates, the filaments return to their normal position. The mouth or pharynx is kept closed,

the anemone remains fully expanded and the products of extracellular digestion are distributed by circulation of the gastrovascular fluid.

3) Food particles are taken up by phagocytosis by endodermal cells in nearly all regions. A few highly specialized cell types, such as trophonemal cells and ciliary tract cells may not be involved.

4) When phagocytosis is complete, the mouth opens and the siphonoglyphs restore a water current through the pharynx to permit the removal of waste material and facilitate gaseous exchange.

A further possibility is that some products of intracellular digestion could be re-distributed by the gastrovascular fluid. These could serve as a nutrient source for those cells which do not carry out significant phagocytosis.

The structure of the trophonema (see Chapter 7) suggests that it is a structure highly specialized for the transfer of nutrients to the growing oocytes or male germ cells. However, it is not clear what proportion of nutrients are supplied by trophonemal cells as opposed to other gonad epithelial cells. Compared to the rest of the gonad epithelium, the trophonemal cells appear to be less active in phagocytosis, perhaps equal in endocytosis, and considerably more active in the incorporation of small molecules. The relative contributions of these different processes to total germ cell nutrition are also not known.

The relatively high level of protein synthesis shown by trophonemal cells compared to the rest of the gonad epithelium raises the interesting possibility that they may

synthesize protein for export to the developing germ cells. In the case of the female gonad, this could mean that a proportion of the yolk protein is synthesized outside the oocyte, by specialized somatic cells.

Chapter 9

OOCYTE BREAKDOWN AND RESORPTION

INTRODUCTION

Oocyte degeneration is a common phenomenon in many groups of animals. Breakdown or atresia of oocytes has been widely reported from mammals (see Byskov, 1978, 1979; Peters and McNatty, 1980) and from several invertebrate groups (see Adiyodi and Adiyodi, 1983a). In spite of its widespread occurrence, very little is known about the mechanisms by which it is brought about or controlled, or the way in which the products of breakdown are resorbed. In particular, ultrastructural studies are scarce even for mammalian species, and there are very few for invertebrates (e.g Diaz, 1979; Eckelbarger *et al*, 1984). The process has not previously been reported from coelenterates.

In the populations of *A. fragacea* sampled during the present study, oocyte breakdown and resorption occurred in most individuals, and at times, especially close to spawning, could affect most of the oocytes in the gonads. This species thus affords an excellent opportunity to observe an important process which has previously received little detailed attention.

RESULTS

Oocytes may break down at any stage during their development, but there appear to be three points in the annual gametogenic cycle when breakdown occurs most frequently. The first peak occurs very early in oogenesis, when the oocytes are less then 12 μm in diameter. The second peak occurs midway through the cycle in December and January, and involves oocytes between 50 and 80 μm in diameter. The third peak occurs among fully grown oocytes at or soon after spawning time.

The first two peaks usually involve relatively small numbers of oocytes, but the third peak of oocyte breakdown may be very extensive, and in some individuals may involve most or possibly all of the large oocytes within the gonads.

1. Breakdown Early in Oogenesis

Among the very smallest oocytes which appear in the endoderm during spring and early summer, a small proportion show signs of degeneration. These signs often include disruption of the nuclear envelope, and the formation of spaces or vacuoles in the nucleus and cytoplasm (Fig. 9.1). The chromatin may appear clumped, and the mitochondria may be more dense than normal. Cells of this appearance have been described in detail as Type III cells in Chapter 4.

Very occasionally, rather larger oocytes appear to degenerate while still within the endoderm. Fig. 9.2 shows an area of endodermal epithelium close to the mesogloea of the gonad which contains what appear to be the remains of a pre-vitellogenic oocyte. There is a space between the endodermal cell bases some 12 μm by 8 μm across. The peripheral region of this space contains large numbers of small membrane-bound vesicles. The central region of the space contains several much larger vesicles. The larger areas contain glycogen, lipid droplets and a few very dense mitochondria. The space between these vesicles is filled with an almost homogeneous matrix of finely granular material of low electron density, which has separated from the larger membrane-bound areas, possibly during fixation. This matrix material is unlike any material found elsewhere in the gonad, but occurs during the degeneration of larger oocytes (see subsequent section) and may be produced

by the breakdown of some oocyte components. The surrounding epithelial cell bases appear normal and undisrupted. It seems likely that this area represents the breakdown products of a small oocyte, perhaps 12 μm in diameter, which has degenerated prior to migration into the mesogloea.

2. Breakdown Midway Through Oogenesis

Many oocytes appear to undergo breakdown at the midway stage of oogenesis, early during compound yolk granule production, at an oocyte diameter of 50-80 μm. Many oocytes reach this size during December and January. Apparently one of the first organelles to be affected during degeneration is the nucleus. No oocytes whose cytoplasm showed any major or general signs of degeneracy were found to contain any vestige of a nucleus. Thus it would appear tha the nucleus breaks down rapidly early in the degeneration process.

Fibrillar granules are a very significant cytoplasmic constituent in most oocytes of this size, and they appear to undergo characteristic changes early during the degenerative process. The limiting membrane around each granule loses its integrity, and neighbouring granules can fuse together. Fusion of fibrillar granules is occasionally seen in healthy oocytes, but never to the extent found during degeneration. The granules are also seen attached to areas of non-fibrillar material of two rather different forms. One form is more dense than the granules themselves, and is rather coarsely granular (Fig. 9.3), while the second form is less dense and more finely granular (Fig. 9.4). Fusion may proceed further to produce extensive confluent areas (Fig. 9.5). These larger areas consist mainly of the less dense material, and it seems

likely that the dense material is an intermediate stage in the conversion of the fibrillar granule contents into the homogeneous, less dense substance.

In most cases, but not all, the mitochondria change markedly in appearance relatively early during degeneration. The mitochondrial matrix becomes very much more dense, and the organization of the cristae and the inner mitochondrial compartment changes (Fig. 9.5). The inner membrane may separate from the outer and form an irregular, twisted tube or series of tubes tangled within the outer membrane sac (Fig. 9.6). In contrast, in a few oocytes, mitochondria of virtually normal appearance may be found even when degeneration of other components is well advanced.

Most oocytes of this size do not contain large numbers of compound yolk granules. Among those that do, however, a proportion of the granules take on an unusual appearance during degeneration. They appear to swell and reduce in density to form large, roughly spherical membrane-bound areas of coarsely granular material, containing numbers of unchanged lipidic inclusions (Fig. 9.7). Most compound granules remain unchanged, however, at least during the early stages.

The cytoplasm may become filled with very large numbers of small membranous vesicles (Fig. 9.8). These tend to be larger (200-500 nm) than the small vesicles found in healthy oocytes, and many contain a wispy fibrillar material. Some, at least, of these vesicles may be derived from the oocyte endoplasmic reticulum.

Many of the changes outlined above involve an expansion and decondensation of normally compact oocyte components. Perhaps as a result of this, there appears to be an increase

in the volume of the oocyte as it degenerates. Often areas of degenerate ooplasm can be found outside the remains of the basal lamina which originally marked the perimeter of the oocyte. These have presumably passed outwards through gaps in the basal lamina (Fig. 9.8). Often no trace of cytospines can be found on the surface of degenerate oocytes, although occasional groups of what may be swollen and distorted cytospines are seen.

Degeneration appears to proceed further by the progressive expansion of the areas of finely granular material presumably derived from the fibrillar granules. These areas may form extensive 'lakes', among which are scattered the vesicularized remains of the other oocyte components (Fig. 9.9). Further evidence that these 'lakes' do derive from fibrillar granules is provided by the finding that fibrillar granules are rarely found among the remaining areas of ooplasm, in spite of their abundance in healthy oocytes of this size. The remaining oocyte components gradually disintegrate, until eventually all that remains of the oocyte is an irregular mass of fine granular material surrounding large numbers of membranous vesicles (Fig. 9.10).

In most cases, the whole oocyte volume appears to undergo degeneration at once, but occasionally oocytes are found which show localized areas of breakdown while the majority of the oocyte seems normal. This may take the form of a small depression in the surface of the oocyte containing small vesicles and other oocyte constituents embedded in finely granular material similar to that described above. Often these appear to move away from the oocyte, and may pass through the basal lamina (Figs. 9.11, 9.12). The oocytes

shown in these two micrographs both contained intact nuclei and otherwise normal ooplasm. Fig. 9.13 shows part of a larger degenerate region, and in this case other small depressions containing finely granular material can be seen close to the large area. The ooplasm is also showing signs of vesicularization, and it may be that this form of localized degeneration can spread and eventually affect the whole oocyte.

Rarely, normal-looking oocytes of this size are found apparently producing blebs from localized areas of their surface (Fig. 9.14). These blebs may be 0.5-1.5 μm in diameter and contain granular cytoplasm, often with a central area of glycogen particles. Less often they contain fibrillar granules, and only rarely other components. Whether bleb formation is and early sign of oocyte breakdown or not is not clear.

3. Degeneration Close to Spawning Time

A very considerable amount of oocyte breakdown may take place around spawning time, during July and August. The amount of breakdown varies between animals and from year to year, but for some animals, the gonads can appear to be filled with degenerate oocytes to the same extent as healthy gonads are filled with normal ones, suggesting that few eggs can have been successfully spawned that season. This may be exceptional, but most gonads show a considerable amount of degeneration at this time.

a) The oocyte nucleus

As with earlier forms of breakdown described above, the oocyte nucleus or germinal vesicle appears to be affected early in the process. In the great majority of oocytes whose cytoplasm appears degenerate, no trace of the nucleus can be

found. On rare occasions, however, what may be the breakdown products of the nucleus can be discerned. These consist of an area within the oocyte free of cytoplasmic organelles but containing a finely granular material (Fig. 9.15). Scattered through this material are small granules of various sizes, and larger dense bodies, which resemble nuclear fibrillar bodies (see Chapter 6, Section A). This region is surrounded by a layer containing numerous annulate lamellae. Most of these are arranged in pairs, although some stacks of three or more are seen. Within the pairs or stacks there is a correspondence in the alignment of the pore complexes. Outside this layer rich in annulate lamellae, a number of membrane-lined clefts are found, arranged in a rough circle. The smaller clefts appear empty, but the larger ones contain numbers of small spherical vesicles, 200-400 nm in diameter. Apart from these, the rest of the cytoplasm in Fig. 9.15 appears normal, suggesting that nuclear changes take place very early in the degenerative process. In oocytes showing other signs of degeneration, membrane-bound 'packets' containing annulate lamellae are sometimes found clustered together (Figs. 9.16, 9.17), which may represent a later stage in the breakdown of a nuclear area.

b) <u>Early cytoplasmic changes</u>

The first sign of degeneration to be noticed in the cytoplasm is the appearance of numerous membranous vesicles, 0.3-0.6 μm in diameter. Many appear empty, while others contain a wispy fibrillar material and some contain smaller vesicles (Fig. 9.18). Two apparently related types of fibrillar material have been found in the ooplasm during the early stages of degeneration which were not seen in healthy

oocytes. The most common form consists of areas of randomly arranged, poorly defined fibrils, dispersed in a finely granular matrix (Figs. 9.19-9.21). The second form consists of irregular areas of fibrils showing some degree of parallel alignment. They may be arranged in separate bundles (Fig. 9.19) or merged into a confluent mass (Fig. 9.20). The two forms are often found together. Fibrillar material may also be found associated with areas of tubular or vesicular material (Fig. 9.21), whose precise nature could not be ascertained.

c) Oocyte fragmentation

The next stage of oocyte breakdown involves the fragmentation of the oocyte cytoplasm into membrane-bound packets. These packets appear to be produced in one of three ways, although these have much in common:

i) Overall fragmentation, in which packets form at approximately the same time throughout the whole oocyte volume,

ii) Centripetal fragmentation, in which fragmentation begins at the periphery of the oocyte and progressively spreads towards its centre,

iii) Peripheral blebbing, in which packets are produced by blebbing from the surface of the oocyte.

i) Overall fragmentation

In this case, fragmentation proceeds by the formation, enlargement and fusion of membrane-lined clefts within the ooplasm, which split it into packets. The clefts appear simultaneously throughout the ooplasm. They do not form randomly, but seem to radiate out from a number of centres or

foci, each of which includes a mass of the randomly fibrillar material described above. It appears that vesicles and tubules become arranged along radii from these masses, and fuse together to from the clefts (Fig. 9.22-9.25). Eventually the clefts give rise to a rosette of cytoplasmic packets, each connected to the central focus by means of a slender process (Fig. 9.26). Fig. 9.27 shows several such rosettes in a small area of ooplasm. From thin sections it is not possible to trace all the connections, but it seems possible that most if not all of the packets seen in this micrograph will be linked by slender processes to fibrillar foci. Eventually the processes become more slender and break (Figs. 9.28, 9.29), and the fibrillar masses disappear, leaving a mass of separate packets.

The packets average some 2-4 μm in diameter, and contain yolk granules, lipid droplets and mitochondria, depending on the composition of the ooplasm prior to fragmentation (Fig. 9.30). Those near the centre of the oocyte area may include annulate lamellae. Immediately prior to fragmentation, the ooplasm often contains large numbers of membranous vesicles, but these are rarely incorporated into the packets. Membranous remnants and vesicles are very commonly found in the spaces between the packets (Fig. 9.30), which may lend weight to the suggestion that the clefts form by a re-arrangement of these vesicles.

ii) <u>Centripetal fragmentation</u>

This proceeds in a similar manner to that described above, but instead of clefts forming simultaneously throughout the ooplasm, they form principally at the surface and cut off the ooplasmic packets to the outside. In many cases, once

the packets have been formed, they move away from the remainder of the oocyte. This produces a central, unfragmented region, separated by a clear space from a surrounding mass of separate packets (Figs. 9.31, 9.32). Although the central mass is unfragmented, it may contain vesicles and clefts, some of which may be incorporated into the packets as they are budded off. It seems likely that further subdivision of the packets may take place after their separation from the central area.

The details of the process by which packets are formed at the surface of the central region are shown in Figs. 9.33 and 9.34. As before, vesicles and tubules become arranged radially around masses of randomly fibrillar material, and clefts form between the surface and these masses. The clefts cut off areas of ooplasm, which then separate. How they move away from the central area is not clear.

iii) Peripheral blebbing

This process is only observed in a small proportion of degenerating oocytes, and it is not fully understood. Membrane-bound sacs, 2-4 μm in diameter, are sometimes found around the periphery of breaking down oocytes. These sacs differ from the ooplasmic packets produced by the above two mechanisms in that their contents do not resemble any part of a healthy oocyte. They rarely contain yolk granules or cytoplasmic organelles, but are filled with sparse, finely granular cytoplasm (Figs. 9.35, 9.36). They are usually found outside the basal lamina surrounding the oocyte area. The early stages of their formation have not been observed, but it seems likely that they arise as outgrowths from the surface of the oocyte rather than by subdivision of the ooplasm. The outgrowths

then separate from the rest of the oocyte, and may move away from it (Fig. 9.37). This process would appear to closely resemble the early stages of breakdown of some eggs soon after spawning, as described in Chapter 13.

d) Composition of the ooplasmic packets

The end product of oocyte fragmentation is that the ooplasm is subdivided into a large number of small, membrane-bound packets, 2-4 μm in diameter (Fig. 9.39). Most cytoplasmic constituents appear to be incorporated into these packets without themselves undergoing any major change in their appearance. Thus packets are found containing compound yolk granules, cortical granules, lipid droplets and mitochondria, all of which seem to be quite normal and identical to those in intact oocytes (Fig. 9.40). Golgi complexes may also be found in packets, although their appearance may be altered (Fig. 9.41).

The vast majority of fibrillar granules are also incorporated into the packets without alteration in their appearance. In a few cases, they lose their limiting membranes and form fibrillar areas after incorporation into packets (Fig. 9,42), and, very rarely, extensive 'lakes' of finely granular material, similar to that seen during degeneration of smaller oocytes, are found (Fig. 9.43). This material may well arise by the fusion and decondensation of fibrillar granules. Why this should occur on a large scale only in a very small proportion of large oocytes is not clear.

Later in the degeneration process, some of the contents of the ooplasmic packets may begin to alter in appearance, before being phagocytosed by other cells. In particular, a

small proportion of compound yolk granules, which generally remain unchanged during fragmentation, may begin to alter. They may lose their spherical shape and become irregular, often curving to enclose areas of the surrounding ooplasm or other granules (Fig. 9.44). The compound granules within a packet may also fuse together, until the entire packet may consist of a mass of compound yolk material, traversed by strands of ooplasm (Figs. 9.45, 9.46). Often a thin rim of ooplasm, 50 nm or so wide, is left around the outside of such packets.

e) Changes at the oocyte surface

Around the periphery of most degenerating oocytes, there is no sign of the cytospines (Fig. 9.47), in spite of the fact that most healthy large oocytes bear numerous cytospines over most of their surface. However, in a few cases, slender cylindrical processes are found around the edge of the area of degeneration. These processes are usually tangled and irregular, and appear to fall into two types (Figs. 9.48, 9.49). The first type are broad, up to 500 nm in diameter and are generally fairly straight. They are filled with a moderately dense, finely fibrillar material. The second type are more slender, usually only 70 nm across, and may extend for 5 μm in section, and so may be considerably longer in three dimensions. They are often undulating or tangled and may show local dilations. They seem to contain a single, central longitudinal strand. Both types have only been found near the edge of degenerating oocytes, and might represent breakdown products of cytospines. Some attach to, or originate from, ooplasmic packets, and an alternative possibility is that at least the more slender processes represent the

remains of the interconnections between the packets formed as they separate.

Healthy oocytes are surrounded by a thin basal lamina, and this also appears to undergo alteration as the oocyte degenerates. The normal basal lamina is some 100 nm thick, and is closely applied to the surface of the oocyte. During breakdown, it tends to separate from the oolemma, and becomes folded and irregular. It also becomes less dense and more floculent in appearance, and thickens, often reaching 250 nm in width. In localized regions, quite large masses of this modified basal lamina material can be found (Fig. 9.50). As degeneration proceeds and the occyte expands, the basal lamina frequently ruptures and ooplasmic packets pass out through it. This may result in lengths of basal lamina being found well back from the edge of the degenerate oocyte (Fig. 9.38).

f) <u>Breakdown of smaller oocytes at spawning time</u>

Smaller oocytes, between 50 and 100 μm in diameter, may occasionally be present in the ovary during the spawning period, and some of these may undergo breakdown along with the fully-grown oocytes. Interestingly, the pattern of degeneration shown by these smaller oocytes is similar to that shown by the large ones, and different from that shown by oocytes of similar size earlier in the year. Thus, these smaller oocytes break up into membrane-bound packets containing intact cytoplasmic constituents, rather than degenerating as a single mass. The packets produced differ from those from larger oocytes, reflecting the different stage of development and cytoplasmic composition of the oocytes. Thus they contain

fewer yolk granules, and some packets contain only granular cytoplasm (Fig. 9.51). Fibrillar granules are an important constituent, and modified Golgi complexes are also common. Occasionally, some packets contain honeycomb-like masses of tubules (Fig. 9.52), which may be derived from endoplasmic reticulum, which is highly developed in normal oocytes of this size. Some packets, however, contain normal looking ER, often arranged concentrically and following the shape of the packet (Fig. 9.53). Rarely, small oocytes degenerating at this time, break up into large, polygonal shaped packets, which may be 10 μm across (Fig. 9.54). These were not found sufficiently frequently to ascertain their mode of formation, however. Some of the events of large oocyte breakdown are summarized in Diagrams 16 and 17.

g) <u>Resorption of oocyte material</u>

When oocytes degenerate midway through their growth period, during the winter months, the breakdown products appear to remain in the mesogloea of the gonad for a considerable time, progressively begoming less and less organized and recognizable. Eventually all that remains is an area of amorphous, finely granular material, in which are scattered membranous vesicles of various sizes (Fig. 9.10). These areas presumably gradually disperse, since no specific mechanism for their removal has been observed. Thus the actual lysis and disintegration of the oocyte organelles and reserve materials takes place extra cellularly, free in the gonad mesogloea.

When oocytes degenerate during the spawning period, however, the situation is very different. The oocyte splits up into small packets, in which the vast majority of oocyte

Diagram 16.

Possible early steps in the fragmentation of large oocytes close to spawning time. The first stage at least in some cases, (left) may be the formation of large, membrane-bound blebs at the surface of the oocyte. The blebs bulge out through the basal lamina and detach from the oocyte. They usually contain only sparse, finely granular cytoplasm, with few or no inclusions. Later,(right) the oocyte cytospines become distorted, and may disappear entirely. Areas of fibrillar material appear in the ooplasm and numerous membranous vesicles and tubules may become arranged in rows radiating from them.

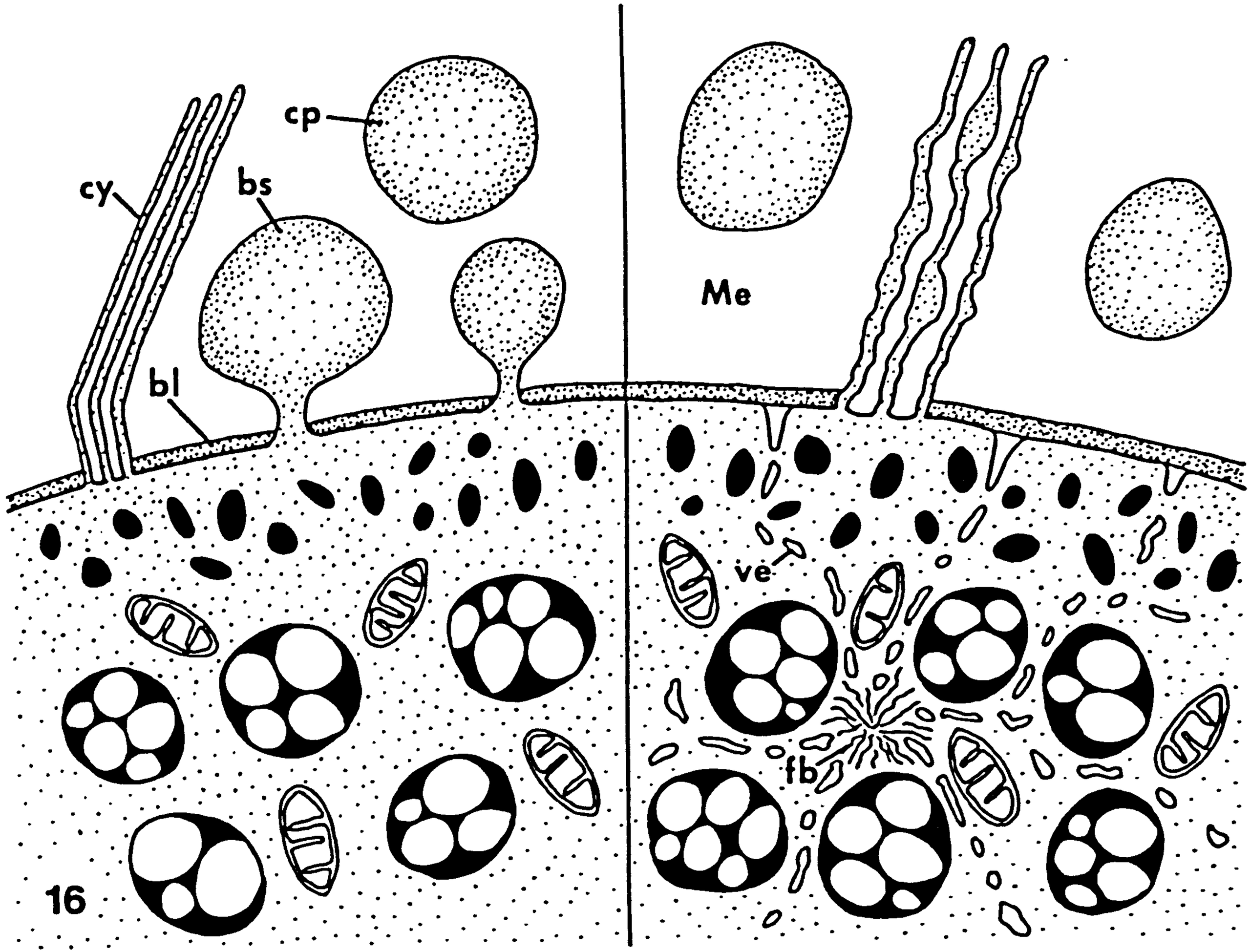
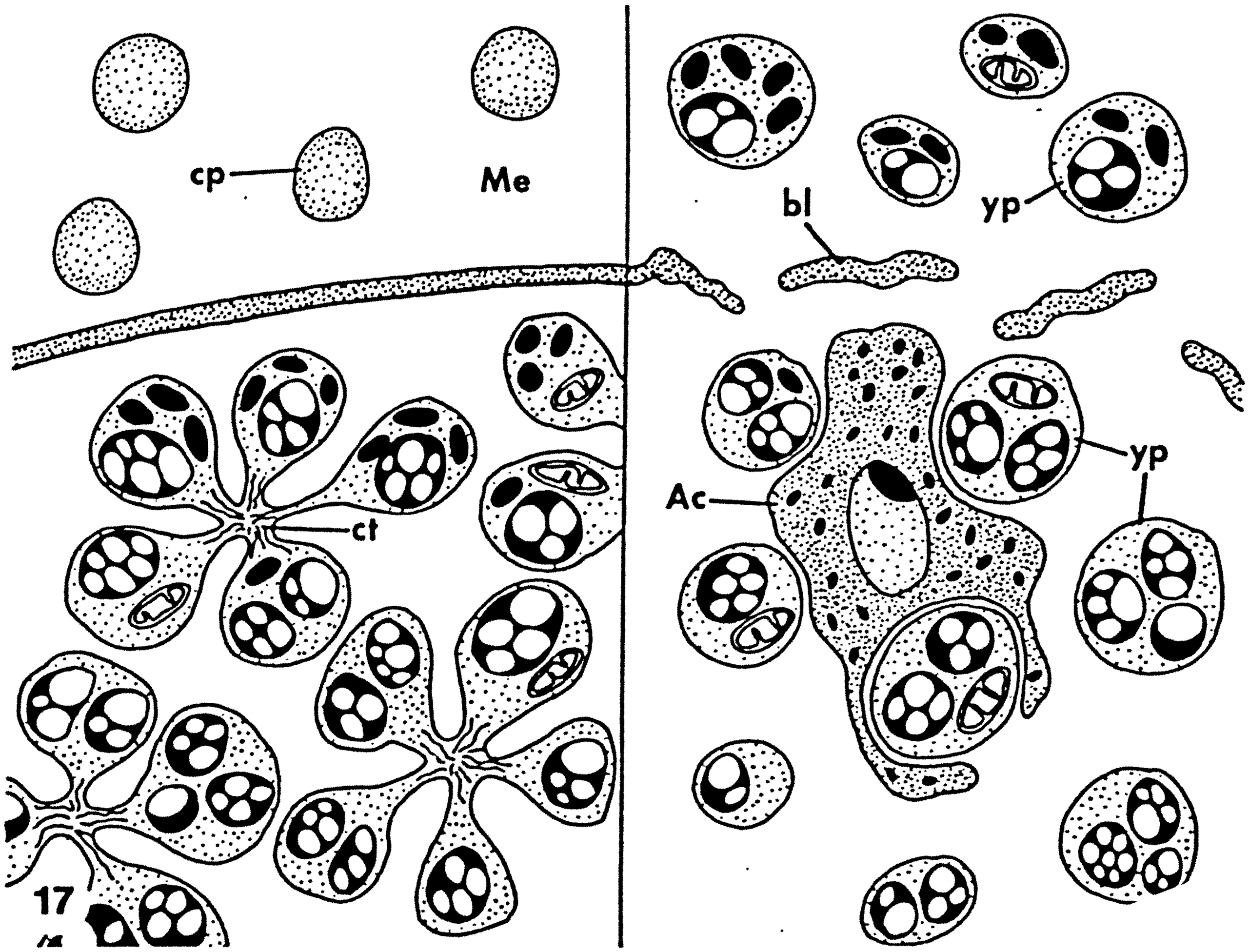

cp
cy
bs
bl
Me
ve
fb
16

Diagram 17.

Possible later stages in the breakdown of large oocytes by overall fragmentation. The ooplasm divides into numerous membrane-bound packets (left), possibly by the fusion of vesicles and tubules. Groups of packets are initially linked by slender stalks to central areas (ct), containing fibrillar material. The packets later separate (right), and move apart from each other. The basal lamina around the oocyte swells and fragments, and many packets may pass out through it. Amoebocytes may invade the area and phagocytose some of the packets. Eventually, most packets break down within gonad epithelial cells.



269.

components remain intact and apparently normal. These packets then appear to be taken up by other, non-germinal cell types, and only after such uptake do the contents of the packets undergo significant degradative changes. Thus the breakdown of the organelles and reserve materials occurs intracellularly and this process involves both the gonad epithelial cells and the granular amoebocytes.

i) Uptake by granular amoebocytes

Granular amoebocytes are common in both the mesogloea and the endoderm of the gonad, and are often found flattened around the surface of developing oocytes (see Chapter 6, Section I). They usually lie in a specific orientation, immediately adjacent to the basal lamina surrounding the oolemma. The amoebocytes never penetrate the basal lamina to contact the oolemma directly (although they do contact the cytospines). In the case of degenerate oocytes, however, amoebocytes are often seen extending through the basal lamina (Figs. 9.55, 9.56), and also, apparently, may pass through entirely. Thus amoebocytes are sometimes found deep inside degenerate oocytes, completely surrounded by ooplasmic packets (Fig. 9.57), and slender processes from the amoebocytes often extend between the packets.

Most amoebocytes found within or around degenerating oocytes contain recognizable oocyte components, which they have presumably phagocytosed. These may include individual yolk granules and entire ooplasmic packets (Fig. 9.58). Occasionally, instances of amoebocytes apparently in the process of surrounding and engulfing such packets are seen. Some of the oocyte remnants within the amoebocytes appear to be undergoing lysis, and residual bodies are sometimes found

in these cells. However, especially in amoebocytes containing large amounts of ooplasmic material, most of it shows no sign of degradation.

ii) Breakdown in gonad epithelial cells

The gonad endodermal epithelial cells in the vicinity of degenerate oocytes may contain large amounts of oocyte-derived material. The basal regions of some cells may be packed with such material, and it may extend into the nuclear zone (Fig. 9.59) or even into the apical regions of the cells (Fig. 9.60). It usually takes the form of membrane-bound packets, 1-3 μm in diameter, which presumably correspond to the ooplasmic packets produced by oocyte fragmentation. Many of these contain clearly recognizable oocyte constituents, most notably compound yolk granules, but also mitochondria and fibrillar granules (Fig. 9.61). Sometimes areas of fibrillar material presumably derived from ruptured fibrillar granules are found (Fig. 9.62). Many of these packets appear to be undergoing breakdown, with distortion and fusion of the compound yolk granules, and some apparently later stages in this process are also found (Fig. 9.63). The terminal stages of this intracellular breakdown of oocyte remnants are thought to be the structures shown in Fig. 9.64. These consist of amorphous, dense granular material surrounding a few small vesicles, with no recognizable structures discernible. The final fate of these remnants is not known.

The amount of intracellular breakdown of oocyte remnants taking place within the epithelial cells would appear to vastly exceed that occurring in the granular amoebocytes. However, the way in which the epithelial cells take up the ooplasmic packets is not obvious. The epithelial cells are

separated from both normal and degenerate oocytes by a layer of mesogloea and two basal laminae, except in the region of the trophonema. The trophonema does provide a region of direct contact with the normal oocyte, but no structures corresponding to trophonemata have been observed associated with degenerate oocytes in spite of the large number examined. The expansion of the oocyte which occurs during breakdown may tend to thin and disrupt the mesogloea around it, and the basal lamina may be altered and discontinuous, but no epithelial cell bases have been observed to pass through these layers and contact the packets. However, amoebocytes often straddle the boundary between oocyte remnant and epithelium, and it is possible that they could 'shuttle' ooplasmic packets from the mesogloea into the endodermal cell layer (Fig. 9.65).

DISCUSSION

The three peaks of oocyte breakdown observed at different stages of the gametogenic cycle may well represent three different underlying processes.

a) The breakdown of very small oocytes while still within the endoderm may be the result of defects incurred during a previous mitosis or during the onset of meiosis. The loss of germ cells during the very early stages of gametogenesis is a widespread phenomenon in invertebrates (e.g Nieuwkoop and Satasurya, 1981), vertebrates (e.g Nieuwkoop and Satasurya, 1979) including mammals (e.g Eddy *et al* 1981). It also occurs during spermatogenesis in *A. fragacea* (see Chapter 11).

b) During the breakdown of early vitellogenic oocytes, signs of degeneration appear in the various cytoplasmic organelles and inclusions before the oocyte as a whole begins to break up. These changes resemble those seen during degeneration or necrosis in a range of somatic cells after injury (Trump *et al*, 1965 and Trump and Ginn, 1969). The products of degeneration remain in place for some time and gradually disperse; no specific mechanism for their removal appears to operate.

c) During the breakdown of fully grown oocytes close to spawning time, the oocyte fragments into membrane-bound packets. The process appears well-ordered, and is initiated before degenerative changes in the organelles and inclusions are apparent. The packets are then phagocytosed by amoebocytes and gonad epithelial cells.

While the first two forms of breakdown may be the result of a failure of some process in normal oocyte development, and so may be regarded as pathoglogical, the last form appears to be a co-ordinated developmental programme in its own right, resulting in the resorption and recycling of oocyte material. The cellular process by which the oocyte breaks into fragments or packets is unusual, and is not similar to the methods of oocyte breakdown found in other groups. One example of a possibly similar mechanism is that of shrinkage necrosis or apoptosis (Kerr, 1971; Kerr *et al*, 1972). This involves the breaking up of cells into a number of membrane-bound, ultrastructurally well-preserved fragments, which are then phagocytosed by surrounding cells. Apoptosis, in contrast to other forms of necrosis, is thought to be an inherently

programmed phenomenon for the controlled deletion of cells. It occurs in a range of situations in normal adult tissues, in embryonic development, and in some malignant tumours. All the cell types reported as undergoing apoptosis have been within the normal size range for somatic cells; it has not previously been found in large cells like the 150 μm diameter oocytes involved here. However, the parallels between apoptosis and controlled oocyte breakdown in *A. fragacea* are striking. Kerr *et al* (1972) point out that the formation of intact, membrane-bound packets during apoptosis prevents release of degradation products into the tissue fluid. These products, released during other forms of necrosis in higher animals, may cause disruption and inflammation of the surrounding tissue. In *A. fragacea*, where oocyte breakdown may occur on a massive scale, such considerations may be very important.

In the few cases where oocyte breakdown has been described in detail from other species, the process appears less well ordered than in *A. fragacea*, and more like conventional necrosis. In the marine sponge *Suberites massa* (Diaz, 1979), the individual oocyte organelles lose their integrity and autophagic vacuoles appear within the oocyte. Eventually the oocytes fragment, but the fragments are only rarely resorbed by phagocytosis. Most fragments are simply expelled from the animal. Diaz concluded that in this species, oocyte degeneration is a pathological process. In those species where oogenesis occurs in close association with accessory cells, these are often also involved in degeneration. In the polychaete annelid *Capitella* (Eckelbarger *et al*, 1984), signs of degeneration are first visible among the organelles within the oocytes. Soon, however, pseudopodial extensions from the

follicle cells invade the cortical regions of the oocytes, and isolate portions of the ooplasm as spherical membrane-bound inclusions. These are then phagocytosed by the follicle cells. This process continues until the oocyte has disintegrated and the follicle cells are laden with inclusions consisting of fused lipid and yolk bodies. The first signs of follicular degeneration or atresia in mammals appear in the follicle cells rather than in the oocyte itself (Byskov, 1979; Peters and McNatty, 1980). There is some evidence that the oocyte remains viable and can be rescued from breakdown if removed from the follicle cells at an early stage (Peluso, 1979).

While somatic cells such as amoebocytes and trophonemal cells are involved in oogenesis in *A. fragacea*, their role during oocyte breakdown may be less extensive than in mammals or *Capitella*. In *A. fragacea*, the fragmentation of the oocyte begins and proceeds apparently quite independantly of other cells. Only after fragmentation is complete do amoebocytes invade the area. Even then, the extent of their role is uncertain. They are undoubtedly phagocytically active, but they are relatively few in number, and the quantity of material they digest may be small. The amoebocytes may serve to disrupt and disperse the accumulations of fragments, and may even pass fragments into the gonad epithelia. However, the majority of resorption and degradation of fragments is carried out within the gonad epithelial cells. Interestingly, Kerr *et al* (1972) report that during apoptosis, neighbouring epithelial cells not normally considered to be phagocytic may become so, and may account for a greater proportion of resorption than specialized phagocytic cells such as histocytes or macrophages.

While the breakdown of small and medium size oocytes in *A. fragacea* may be pathological, the resorption of large oocytes appears to be a programmed process and may be adaptive. Oosorption may provide a mechanism by which the output of the gonad may be regulated in response to changing circumstances. In this way, energy originally allocated to gamete production may be re-directed back into stored energy available for somatic growth (Eckelbarger *et al*, 1984). This may be of benefit if external conditions are such that the chance of survival of the progeny is low, or if the survival of the adult is threatened unless this energy is made available to it. Olive *et al* (1981) argue that oosorption on a large scale is inappropriate for species with a short life span. Obviously, oosorption is of little value to a short-lived animal capable of spawning only once. But for animals with longer life spans, and with the capability of several gametogenic and spawning cycles, oosorption may, under certain circumstances, be an advantageous tactic.

Sea anemones are generally thought to be long-lived animals. Ashworth and Annandale (1904) describe specimens of *Cereus pedunculatus* which have been maintained in captivity for over 65 years. Stephenson (1928) considers that, in the wild, anemones can live for hundreds of years under suitable conditions. Field studies have not been undertaken for *A. fragacea*, but for *A. equina*, Rees (pers. comm) found that juvenile mortality was very high and adult maortality very low at two British sites. More precise information is available for *A. tenebrosa* (Ottaway, 1981). Based on estimates both of annual growth rates and adult mortality, Ottaway concluded that mean longevity in a natural population is some

50 years, with some individuals living as long as 210 years. During the present study, only three small (less than 20 mm pedal disc diameter) *A. fragacea* individuals were found during three years of regular sampling from two different sites. This suggests that the level of juvenile recruitment into the population is very low, which in turn suggests that adult mortality is also very low. It thus seems likely that *A. fragacea* is similar to some other anemones, and may live for perhaps several decades. Stephenson (1928) indicates that there seems to be no decline in reproductive activity with increasing age, and all the *A. fragacea* individuals collected during the present study contained gonads.

It thus seems likely that most *A. fragacea* individuals undergo many gametogenic cycles during their lifetimes. Under these circumstances, oocyte resorption may become an important option in the reproductive strategy. As expressed by Calow (1984), when extrinsic mortality risks are high for parents, then, in general, it pays to take risks to produce offspring. Conversely, when parental mortality risks are low, and it seems likely that in some sea anemones they are very low, it pays to keep investment in reproduction to a minimum. This may be especially true in conditions of high population density (Olive *et al*, 1981).

If it is advantageous for *A. fragacea* to put relatively little effort into reproduction, and as seems likely, most oocytes are resorbed each year, it may seem surprising that such large scale gametogenesis is initiated each year. However, resorption of fully grown oocytes may have some advantages over reduction of oocyte numbers at the start of the cycle. For a species like *A. fragacea* where egg

development takes a long time, perhaps over a year, oosorption enables the final decision as to how many eggs are to be released to be delayed until just prior to spawning.

The combination of slow oocyte development and extreme longevity may make oosorption a particularly important process for sea anemones. Anemones may also prove to be highly suitable organisms for the future study of the phenomenon. Oosorption similar to that seen in *A. fragacea* was also observed in *A. equina*, *Cereus pedunculatus* and *Anemonia sulcata* (see Chapter 10). A similar fragmentation mechanism also appears to operate during degeneration of some eggs after spawning, and during blastocoele infilling in normal development (see Chapter 13).

The presence of oocytes degenerating in various ways calls for caution in the interpretation of micrographs. Only by the detailed examination of a range of stages can structures be identified as normal or degenerate with any confidence. Oocyte degeneration was found to be widespread in *A. fragacea* and other species during the present study, but has not been identified as such in previous studies. It may be that some features described as normal by some authors are in fact features of degenerate oocytes. For example, the description of *A. equina* oocytes surrounded by small vesicles and vacuoles and material 'rinsing' through the mesogloea given by Schmidt and Schäfer (1980) is not consistent with the healthy oocytes seen in the present study, but could apply to degenerate ones. Their Figure 1 would also appear to show a degenerate rather than a normal oocyte. The misinterpretation of the process of degeneration may lead to more serious

difficulties. Schmidt and Schäfer (1980) observed yolk packets in endodermal epithelial cells, and concluded that they were indicative of extra-oocytic yolk formation, and that intact yolk bodies could be transferred to the growing oocytes. On the basis of the present study, it seems much more likely that these endodermal yolk bodies have originated from degenerate oocytes, and it appears that transfer of complete yolk bodies into oocytes does not occur. Thus an awareness of the process of breakdown may be important for the correct interpretation of many features of oocyte development.

difficulties. Schmidt and Schäfer (1980) observed yolk packets in endodermal epithelial cells, and concluded that they were indicative of extra-oocytic yolk formation, and that intact yolk bodies could be transferred to the growing oocytes. On the basis of the present study, it seems much more likely that these endodermal yolk bodies have originated from degenerate oocytes, and it appears that transfer of complete yolk bodies into oocytes does not occur. Thus an awareness of the process of breakdown may be important for the correct interpretation of many features of oocyte development.

Chapter 10

OOGENESIS IN OTHER SPECIES OF ANEMONE

INTRODUCTION

In addition to *Actinia fragacea*, oogenesis was also investigated by regular sampling in *Actinia equina*, *Cereus pedunculatus*, *Tealia felina* and *Anemonia sulcata*. The sampling procedures for these species are outlined in Chapter 2. At no stage was it intended that these species should be studied in the same detail as *A. fragacea*, but it was thought worthwhile to examine some other species for comparative purposes. *Cereus pedunculatus*, in particular, might provide an interesting comparison, since it is taxonomically separated from the other anemone species studied, and inhabits a very different environment. Previous work had also shown that it could employ a variety of reproductive strategies in different locations (Rossi, 1974). *Actinia equina* also shows a different reproductive strategy from *A. fragacea*, since it broods its young (Chia and Rostron, 1970; Cain, 1974; Carter and Thorpe, 1979; Gashout and Ormond, 1979; Carter and Thorpe, 1981). While the mechanism of reproduction in *A. equina* is still controversial, it was thought it might provide an interesting contrast with the closely related *A. fragacea*.

In fact, there appear to be very large areas of similarity in the process of oogenesis between all the species studied, and so a detailed description of oogenesis in each species would prove to be repetitive. Thus, in the following description, only those areas which either

a) Differ markedly from corresponding processes in *A. fragacea*, or

b) Clarify or confirm areas which remained unclear or controversial in the studies of *A. fragacea*, will be described

in any detail. Overall, *Cereus* provided perhaps the most interesting comparison, and so will be dealt with most thoroughly. Only specific aspects of the other species will be considered.

RESULTS

1. *Actinia equina*

Cytologically, all stages of oogenesis in *A. equina* were found to be very similar to those already described for *A. fragacea*. The appearance of most of the oocyte organelles and inclusions appears identical in both species (Fig. 10.1). Some points of difference did emerge.

a) Only approximately one quarter of large *A. equina* individuals in the population studied contained recognizable gonads, compared with 100% for *A. fragacea*. It also seemed that each individual contained fewer, smaller gonads than found in *A. fragacea*, although this was not quantified.

b) The ooplasm of vitellogenic *A. equina* oocytes contains large numbers of small membranous vesicles and tubules. These are similar in size to those found in *A. fragacea*, but tend to be more regular in shape and smoother in outline. They are common in central regions close to the nucleus (Fig. 10.2) as well as in the peripheral ooplasm.

c) Fibrillar granules were not observed in *A. equina* oocytes at any stage of their development. The cortical regions of some large oocytes contain a small number of irregular vesicles containing material of moderate electron density, but with no trace of a fibrillar substructure (Fig. 10.3).

d) Low density granules were only rarely found in the peripheral ooplasm of *A. equina* oocytes.

The structure of the trophonema, the relationship between oocytes and granular amoebocytes, and the mechanism of fragmentation and resorption of large oocytes were all similar in both species. No instances where apparently all the large oocytes in a gonad underwent resorption were observed in *A. equina*, however.

2. *Cereus pedunculatus*

The surface of the gonad epithellium in *Cereus* appears to conform to the outline of the developing oocytes more than in *A. fragacea*, so the female gonads resemble mulberries or bunches of grapes rather than smooth sheets. The gonads are usually dark brown in colour, presumably due to the presence of numerous zooxanthellae.

Examination of samples from varous times of year by light microscopy suggests that the gametogenic cycle, if it exists at all, is much less clearly defined than in *A. fragacea*. Oocytes of all sizes were found at all times of year, and no peak period of small oocyte formation could be discerned. The sex ratio of the anemones sampled showed a great preponderance of females. Out of over 80 anemones sampled, one was hermaphrodite, showing oocytes and testicular cysts within the same gonad, and one appeared to be exclusively male. All the remainder contained female gonads. However, only large anemones were collected, and only a small number of gonads from each anemone were examined histologically, so this situation may not be true of the population as a whole.

The fully grown oocytes are slightly smaller than those of *A. fragacea*, reaching 120-140 μm in diameter.

The ultrastrucutral description of oogenesis has been divided into the following six sections:

a) Previtellogenic stages

b) Oocyte growth and vitellogenesis

c) Uptake of other cells by oocytes

d) Trophonemal structure

e) Oocyte breakdown and resorption

f) Algal symbionts.

a) <u>Previtellogenic stages</u>

The smallest recognizable female germ cells found in *Cereus* are broadly similar to those described for *A. fragacea* (see Chapter 4). They are small rounded cells, 5-7 μm in diameter, with relatively large nuclei, and occur among the bases of the gonad epithelial cells, close to the mesogloea. As in *A. fragacea*, the nucleus is generally of very low electron density, with wispy, finely fibrillar material, small nuclear granules, and usually a single granular nucleolus lying in the electron lucent nucleoplasm (Figs. 10.4-10.6). Unlike *A. fragacea*, however, the cytoplasm of these small germ cells is also of low density, often appearing no more dense than that of the surrounding epithelial cells. This lower cytoplasmic density appears to be the result of smaller numbers of free ribosomes. Otherwise the organelle content is similar to corresponding cells in *A. fragacea*. Nuage material was seen in all these small cells, and will be described below. The outline of these cells may undulate, but is generally very smooth, and the membranes of the surrounding cells, both other

germ cells and epithelial cells, follow the cell contours closely (Figs. 10.4, 10.5). Usually the intermembrane gap is narrow, some 25 nm, and very constant around most of the germ cell surface. No small vesicles or other materials are normally found around the germ cells, and they bear no extracellular coat of any description.

A significant difference from the situation in *A. fragacea* is that these smallest female germ cells often occur in groups rather than being scattered through the gonad. They may be aligned in rows bordering the mesogloea (Fig. 10.6) or may occur as nests of various sizes within the endoderm (Figs. 10.7, 10.8). There is evidence of mitotic activity among the cells within these nests. Some cells show no sign of a nuclear envelope, and their chromatin may appear as irregular, dense granular areas, very different from the diffuse nuclear material found in cells with a nuclear envelope. Since not all the cells within a nest may appear in this condition, it appears that mitosis is not synchronous. Cytoplasmic bridges have not been observed between the cells of a nest, although their membranes lie closely adjacent.

Slightly larger endodermal germ cells, between 8 and 15 μm in diameter, are thought to be oocytes. They have a more dense nuceleus than the smallest cells, with irregular patches of dense chromatin, small nuclear granules and a nucleolus (Figs. 10.9-10.11). Rarely, two nucleoli may be present, and structures resembling synaptinemal complexes are occasionally found (Fig. 10.12). No signs of mitosis or nuclear envelope disruption were found in these larger cells. The cytoplasm remains of low density although its organelle content increases. Mitochondria may be quite numerous

(Fig. 10.10) and may be arranged around the nucleus. Some may have prismatic cristae. Long individual cisternae of endoplasmic reticulum are found (Fig. 10.10) and a Golgi complex may be visible (Fig. 10.11). Lipid droplets are common, but amounts of glycogen in the cytoplasm tend to be lower than for corresponding cells in *A. fragacea*. Occasionally cytoplasmic axonemes were found (Fig. 10.11). The nuage material appears slightly different from that in *A. fragacea* oocytes, and again may take on a number of forms. Many oocytes contain clusters of small dense bodies, each some 60 nm in diameter, some of which appear hollow or tubular (Fig. 10.13). Often areas of finely granular dense material surround or are intermingled with less dense, more fibrillar structures, in a similar arrangement to that of *A. fragacea* (Figs. 10.14, 10.15), but rather less regular. Small areas of dense, granular cytoplasm identical to those found in *A. fragacea* are also common (Figs. 10.12, 10.13). Several areas of nuage, often of different types, may be seen in a section of a single oocyte. Overall, nuage would appear to be more abundant in *Cereus* oocytes than *A. fragacea* oocytes of similar size.

As the oocytes enlarge further in the endoderm, the electron density of both nucleus and cytoplasm increases, the latter apparently due to greater numbers of free ribosomes (Fig. 10.16). No oocytes larger than about 20 μm diameter were found in the endoderm, so it seems likely that entry into the mesogloea usually occurs before that stage.

No fibrillar or compound yolk granules were observed within the endodermal oocytes, so it appears that vitellogenesis normally begins after entry. The process of entry into the mesogloea was not observed.

b) Oocyte growth and vitellogenesis

Once the oocytes are within the mesogloea, the phase of vegetative oocyte growth and vitellogenesis begins. The nucleus increases in size and reduces in density to resemble that of vitellogenic *A. fragacea* oocytes (Fig. 10.17). Initially, smaller nuclear granules are common, but they are found less frequently in large oocytes. The nucleolus becomes more discrete and enlarges. Unlike in *A. fragacea*, it may contain vacuoles of less dense material resembling the surrounding nucleoplasm (Fig. 10.18). Nuclear fibrillar bodies, identical to those of *A. fragacea* are commonly found, usually near the periphery of the nucleus (Fig. 10.19). The outline of the nuclear envelope is generally undulating, although in one oocyte it was seen to be highly folded (Fig. 10.20). Whether this is a normal condition is not known. Large numbers of mitochondria may accumulate in the perinuclear ooplasm in the early stages of vitellogenesis (Fig. 10.21), and nuage material is often associated with these accumulations. Extensive mitochondrial clouds like those of *A. fragacea* have never been observed.

Rough endoplasmic reticulum is found usually as individual cisternae distributed throughout the ooplasm. Occasionally, concentric whorls of ER cisternae are found (Fig. 10.22) as in *A. fragacea*. At no time were massive, highly ordered perinuclear accumulations of rough ER observed in *Cereus* oocytes. Stacks of annulate lamellae are common (Fig. 10.23), and these also show no preference for a perinuclear location, but may be found throughout the cytoplasm. Most seem to be independant of any ER, but occasionally short

lengths of ER may extend from the ends of the annulate lamellae (Fig. 10.24). Lipid droplets are common in the ooplasm of larger oocytes, and some may be surrounded by single RER cisternae (Fig. 10.24). Glycogen is also found, but usually as small deposits (Fig. 10.25). Massive glycogen deposits have not been observed in *Cereus* oocytes.

i) Fibrillar granules

Fibrillar granules are the first oocyte- specific granules to appear in *Cereus* oocytes, as they are for *A. fragacea*. As in *A. fragacea*, they are membrane-bound and contain roughly parallel bundles of fibrils, each of which is X-shaped in cross-section (Fig. 10.26). The dimensions of the fibrils are similar in both species. In *Cereus* oocytes, 'low-density' or 'loosely packed' granules are only rarely seen; when fibrils are present, they are usually regularly arranged. However, many of these granules in *Cereus* contain non-fibrillar regions. Very often, there is a single, roughly central region containing well defined and regularly arranged fibrils, while the remainder of the granule contains a granular material with no sign of a fibrillar substructure (Fig. 10.27). No granules containing more than one fibrillar region have been found, and no intermediate material between the granular and the fibrillar arrangements has been seen. Granules containing identical granular material but apparently lacking fibrillar regions are quite common, however (Fig. 10.28). These granules are often associated with Golgi complexes, and may represent a stage in the formation or maturation of the fibrillar granules (see Discussion). In *Cereus*, most fibrillar granules, including large ones, contain fibrils aligned, if only approximately,

along a single axis; it may be that fusion of granules already containing orientated fibrils is less common than in *A. fragacea*. As in *A. fragacea*, in nearly fully-grown oocytes, the fibrillar granules come to occupy the peripheral regions of the ooplasm.

ii) Compound yolk granules

In *Cereus*, as in *A. fragacea*, compound yolk granule formation begins later than fibrillar granule formation. They also are characterized by containing a number of spherical, electron lucent inclusions, probably lipid droplets, embedded in an electron dense matrix. However, they are highly variable in appearance. A relatively small proportion closely resemble the most common *A. fragacea* granules in having a small number of relatively large lipid-like inclusions. In *Cereus*, however, most yolk granules contain large numbers of very small inclusions, and there appears to be a continuous spectrum of granules containing inclusions of decreasing size, leading to granules of the vesicular type (Fig. 10.29). In these vesicular granules, the component vesicles may show signs of a regular arrangement (Fig. 10.30). Many *Cereus* compound yolk granules contain large areas of what, at low magnification, appears to be homogeneous dense material. These areas may appear solid and straight-sided, or they may occupy the periphery of the granule (Fig. 10.31). At high magnification, this dense material may display a regular para-crystalline lattice sub-structure. In Fig. 10.32, the granule appears to contain two regions of dense material, with the lattice orientation in different planes. Areas of homogeneous dense material may also be found within vesicular granules.

Another class of granule, which does not appear to correspond to any type seen in *A. fragacea* has been found in

Cereus oocytes, although much less commonly than those described above. These granules may be 1-2 μm in diameter, and may be elongate or angular rather than spherical (Fig. 10.33). They contain material of moderate electron density, which is much more coarsely granular than the granular component of some fibrillar granules. They also contain a small number of lipid-like inclusions and very small dense particles. These granules appear similar to those described by Schäfer (1983), which he called primary yolk granules.

iii) Cortical granules

Cortical granules appear in the cytoplasm relatively late during oogenesis. They are similar in size and appearance to those described for *A.fragacea*, but seem to be of slightly lower electron density, and some may be more irregular in shape (Fig. 10.34). Perhaps because of their lower density, a band of more dense material is visible, just beneath the limiting membrane, which is not obvious in *A. fragacea* cortical granules (Fig. 10.35). This layer may be 20-50 nm wide, varying in thickness around the granule. Otherwise, the granule contents appear homogeneous. Golgi complexes associated with what appear to be smaller versions of cortical granules are common in the peripheral ooplasm (Figs. 10.36, 10.37).

Low density granules may be found immediately beneath the oolemma, although perhaps less commonly than in *A. fragacea*. They usually contain finely granular material in varying degrees of expansion (Fig. 10.38). Some contain material identical to the granular component of fibrillar granules, and as with *A. fragacea*, it is not clear whether they arise from cortical

granules or fibrillar granules. Sometimes, these granules are surrounded by a layer of finely granular material which is not membrane-bound, and whose significance is unclear (Fig. 10.38).

iv) <u>The oocyte surface</u>

The surface of large *Cereus* oocytes bears a rather sparse covering of microvilli. These appear to be much smaller than the cytospines on the surface of *A. fragacea* oocytes, and are nor organized into characteristic tufts. Most are 80-100 nm in diameter, and they usually lie folded against the surface of the oocyte (Fig. 10.39). Very few have been found sectioned in accurate LS, so it is difficult to estimate their length. However, none have been observed extending for more than 3 μm, compared with the 12 μm cytospines regularly found in large *A. fragacea* oocytes. They do not appear to have a well-developed fibrillar core, and only rudimentary rootlets have been observed extending from their bases. Broader and less regular processes are also found extending from the oocyte surface (Fig. 10.39). Whether these represent expanded microvilli or are produced in another way is not known.

The oocyte surface may be indented or invaginated in several ways. Broad, shallow depressions, up to 300 nm across, are frequently found (Fig. 10.39). Typical, bristle coated pinocytotic pits are also common and can occur anywhere on the oocyte surface, although they are never present in large numbers (Fig. 10.40). Less commonly, deeper clefts may be found in the surface. These may be 1-3 μm deep, and may be relatively wide and contain microvilli, or they may be very narrow. An unusual, bifurcated cleft is shown in Fig. 10.41. At the

bottom of each narrow cleft is an expanded region, where a layer of finely fibrillar material lies immediately beneath the oolemma. The significance of these clefts is unknown.

Mesogloeal oocytes are covered by a layer of basal lamina material which appears thicker and more diffuse than that found in *A. fragacea* (Fig. 10.42), averaging some 150 nm in thickness. It is, however, very similar to the basal lamina of the gonad epithelial cells. Granular amoebocytes are occasionally found close to the surface of mesogloeal oocytes, but do not appear to flatten around them or enter into a close or precise relationship with them as was noted in *A. fragacea*. Rarely, non-germinal cell processes are found contacting the oocyte surface. These may contain glycogen and lipid droplets, and lie closely against the oolemma (Fig. 10.42). The intermembrane gap is only some 20 nm, and the basal lamina lies outside the processes. It is not certain whether these processes extend from amoebocytes or gonad epithelial cells.

c) Uptake of Small Cells by Oocytes

Many large *Cereus* oocytes are seen to contain one or several complete small cells. They normally occur in the cortical regions of the oocytes, but are usually completely surrounded by ooplasm. Most oocytes containing these cells are large and already contain cortical granules, so the uptake of small cells would appear to be a feature of the late vitellogenic period of oocyte growth.

The cells found within oocytes are always small, some 5-10 μm in diameter, with relatively large nuclei. Both nucleus and cytoplasm are of generally low electron density (Fig. 10.43)

and in appearance and organelle complement these cells resemble early oocytes of comparable size described earlier in this chapter. Larger included cells appear more dense than the smallest, and may contain glycogen, lipid droplets and numerous mitochondria. Many included cells contain material resembling the nuage material of early female germ cells, (Fig. 10.44), and overall it seems likely that these included cells are oogonia or early oocytes.

The plasma membrane of the included cell is closely surrounded by another membrane, presumably derived from the oolemma of the surrounding oocyte. The two membranes show a remarkably constant intermembrane gap of some 20 nm over virtually the whole cell surface (Figs. 10.44, 10.45). Occasionally, double membrane loops and whorls may extend out from the surface of the enclosed cell (Fig. 10.44). Sometimes the included cells may contain apparently empty areas in the cytoplasm which might indicate the onset of degeneration, but no advanced stages of degeneration have been seen. Often two included cells may be found close together in the peripheral ooplasm of a large oocyte (Fig. 10.46).

These small cells appear to be taken up by large oocytes by a process resembling phagocytosis. Small cells are occasionally seen apparently in the process of being surrounded and engulfed by large oocytes. The regions of the large oocyte involved may have a very irregular outline, with large out-growths resembling pseudopodia (Fig. 10.47), and seem to be more richly endowed with microvilli than the remainder of the oocyte.

Initial contact between the large oocyte and the cells destined for uptake is presumably made by the oocyte microvilli.

Occasionally small cells are seen in depressions in the surface of large oocytes, and the microvilli may appear to be pressed closely around the surface of the small cells (Fig. 10.48). The very close membrane apposition between the two cells noted above is already apparent before the process of uptake is complete (Fig. 10.49). The early stages of uptake have not been observed, but the small cell must in some way penetrate the basal lamina around the large oocyte before membrane contact can be established. Sometimes small cells still in the process of being engulfed already appear degenerate (Fig. 10.50).

d) The Trophonema

Cereus oocytes, like those of *A. fragacea*, develop in association with a trophonema. This structure is basically similar in the two species, but there appear to be some differences, which will be considered briefly here. In both cases, the trophonema consists of a gap or pore in the mesogloea around the oocyte, through which direct contact between the oocyte and the gonad epithelial cells can take place. In *A. fragacea*, and in most cases in *Cereus*, the epithelial cell bases project through the pore and terminate in a depression in the surface of the oocyte (Fig. 10.51), and the oocyte nucleus is displaced towards the trophonema. In *Cereus* however, the area of contact between oocyte and endoderm appears to be less specialized. In particular, the depression in the oocyte is generally devoid of microvilli, although these may be common on the remainder of the oocyte surface. Close membrane contact between oocyte and endodermal cells does occur, but extensive intercellular junctions do not form

between them. As in *A. fragacea*, bundles of microfilaments are common in the trophonemal cells, especially close to the lip of the pore in the mesogloea (Fig. 10.52). The ooplasm immediately beneath the area of contact also appears less specialized than in *A. fragacea*. It may contain small membranous vesicles, but never in great numbers, and it often contains cortical and fibrillar granules and compound yolk granules, much as the rest of the ooplasm.

A number of trophonemata in *Cereus* were found in which, rather than the epithelial bases projecting into a depression in the oocyte, the oocyte protruded out through the pore in the mesogloea (Fig. 10.53). Close membrane contact between oocyte and endoderm still occurs in this region, and limited intercellular junction formation may take place (Fig. 10.54). Interestingly, even in these cases of oocyte protrusion, epithelial cell bases may still project through the mesogloeal pore around its periphery, spread out beneath the mesogloea and contact a shallow depression in the oocyte (Figs. 10.54, 10.55). The ooplasm within these protrusions may contain lower concentrations of granules and organelles than the remainder of the oocyte, but is still much more normal in appearance than that associated with trophonemata in *A. fragacea*.

e) <u>Oocyte Breakdown and Resorption</u>

The degeneration and resorption of oocytes within the gonad was frequently observed in *Cereus*, although never on the scale sometimes found in *A. fragacea*. The pattern and mechanism of breakdown appears similar in the two species, and so will be described only briefly here. As in *A. fragacea*, oocytes appear to break down in one of two ways, apparently

depending on their size.

Small and medium sized oocytes (up to some 70 μm in diameter) undergo breakdown by a process in which many components lose their integrity and coalesce, giving rise to extensive areas of finely granular material. The remainder of the ooplasm becomes vesiculated and chaotic (Fig. 10.56). The oocyte microvilli are apparently not lost very early in this process, since swollen and distorted remnants may be recognized around the periphery of obviously degenerate oocytes. Usually the integrity of the nucleus is lost early during breakdown, but rarely an apparently intact nucleus and nuclear envelope may be seen in a degenerate oocyte, as in Fig. 10.57. However, the nucleus in this micrograph contains numerous small membranous vesicles, and a structure resembling a nucleolus is seen outside the nuclear envelope. It appears that while a nucleus is obviously present, it may be abnormal.

Large oocytes may undergo breakdown by a process of fragmentation forming small, membrane-bound packets. The contents of these packets are usually unchanged in appearance from corresponding structures within healthy oocytes. The fragmentation process may proceed from the periphery of the oocyte towards its centre (Fig. 10.58). The ooplasmic packets are taken up by the endodermal epithelial cells, where final degradation of their contents takes place (Fig. 10.59). The extensive involvement of granular amoebocytes in the resorption of the ooplasmic fragments, as noted for *A. fragacea*, was not observed in *Cereus*.

f) Symbiotic Algae

The gonad epithelial cells contain large numbers of symbiotic algae, which give the gonads a characteristic brown colouration. This permits their easy discrimination from the surrounding mesentery and filament tissue, which contains few algae and is much paler in colour. The algae are found at all levels in the epithelial cells, from gonad surface to junction with the mesogloea (Figs. 10.60, 10.61).

The algae are enclosed within membrane-bound vacuoles within the gonad epithelial cells. Occasionally two algal cells are found within a single vacuole, but this is probably indicative of recent cell division (Fig. 10.62); usually there is only a single cell in each vacuole. The algae appear to be typical dinoflagellate zooxanthellae, very similar to those described by Taylor (1968) from *Anemonia sulcata*. For this reason, they will not be described in detail here. Important features of these cells include the peripherally located chloroplast, the nucleus with dense chromosomes, and the dense, partly mineralized accumulation body (Figs. 10.63, 10.64). An important taxonomic feature is the projecting pyrenoid, surrounded by a thick starch sheath. The contents of the pyrenoid are seen to be continuous with the stroma of the chloroplast (Fig. 10.64). The cytoplasm also contain mitochondria, starch grains and crystals of calcium oxalate. The chloroplast lamellations can be seen to consist of three closely associated thylakoids (Fig. 10.65). Most of the algal cells seen in *Cereus* gonads were 7-8 μm in diameter; this is considerably smaller than Taylor's (1968) estimate of 12 μm in *Anemonia*.

Algal cells apparently in various stages of degeneration were also found in gonad epithellal cells, often alongside normal-seeming algae (Figs. 10.66, 10.67). It thus appears that the algae can pass through several stages of their life cycle while within the gonad.

Algal cells were never observed within oocytes at any stage. They were also not found within trophonemal cells or in the mesogloea, or in any form of association with the developing oocytes. How the symblonts are passed to the progeny is not known.

3. *Tealia felina*

The fully grown oocytes of *Tealia felina* are considerably larger than those of *A. fragacea*, reaching some 600 μm in diameter. The gonad epithellal cells are only perhaps 50 μm in height, and the intact female gonads resemble clusters of grapes in appearance (Fig. 10.68). No annual cycle was apparent from the sectioned material; large and small oocytes were present in all the samples. All the large individuals dissected contained gonads. The sexes appear to be separate, and were found in approximately equal numbers. Unlike in the other species studied, the large oocytes of *Tealia felina*, if teased out of the gonad, are positively bouyant. The smaller oocytes resemble the early stages of *A. fragacea*, but the larger oocytes (>150 μm) show some differences, and will be described briefly.

The oocyte nucleus usually appears more dense than the surrounding cyoplasm, and may reach 130 μm in diameter. It often contains one large and several smaller nucleoli. The cytoplasm is dominated by large spherical bodies which

appear empty in light microscope sections (Fig. 10.69), but which under EM are seen to be lipid droplets (Fig. 10.70). They are usually homogeneous, and may reach 15 μm in diameter. They are generally absent from the cortical ooplasm, but are otherwise evenly distributed, and may indent the nuclear envelope (Fig. 10.69). They sometimes display a more dense outer region (Fig. 10.71) but this may be the result of extraction, or incomplete penetration by osmium tetroxide, rather than a change in the nature of the lipid material.

The compound yolk granules appear to be of two different kinds, although they may be related. The more common type of granule is 1-2 μm in diameter, and consists usually of one, but occasionally two or three, large lipidic inclusions embedded in an electron dense matrix (Fig. 10.71). The inclusions are often situated eccentrically, giving the granule a flask-like shape, and they may contact or even penetrate the outer membrane. The inclusions may show a more dense peripheral band as found around some large lipid droplets. The dense matrix material may appear homogeneous, but it often contains fibrillar arrays (Fig. 10.72), hoops of dense material coiled around the inclusion (Fig. 10.73) or myelinic membrane whorls (Fig. 10.74). The less common form of compound yolk granule resembles those of *A. fragacea* in containing numerous, smaller lipidic inclusions (Fig. 10.75).

The cytoplasm also contains numbers of granules containing a finely granular material of moderate density (Fig. 10.75). Sometimes granules of lower density are found, which may represent a less condensed form of these granules.

The peripheral ooplasm contains mitochondria and fibrillar granules of various packing densities (Fig. 10.76).

Some fibrillar granules contain non-fibrillar regions, whose contents resemble those of the moderate density granules described above (Fig. 19.77). Dense cortical granules occur close to the oolemma. The relationship, if any, between fibrillar granules, moderate density granules and cortical granules is uncertain, but their contents, on morphological grounds, could all be different condensation states of the same material.

The oocyte surface bears cytospines arranged in tufts, like *A. fragacea*. In large tufts, the cytospine rootlets may appear to merge into a large mass in which individual rootlets cannot be distinguished (Fig. 10.76). Smaller tufts show individual rootlets, however (Fig. 10.77).

Amoebocytes were not observed closely associated with oocytes. The structure of the trophonema seems similar to that of *A. fragacea*, and involves the oocyte cytospines. No signs of the fragmentation of large oocytes was observed by electron microscopy. Using the light microscope, basophilic lobes were seen around the periphery of a small proportion of large oocytes in a sample taken during August. These might be indicative of oocyte breakdown, but the mechanism could not be ascertained.

4. *Anemonia sulcata*

Only large *Anemonia sulcata* individuals (with a pedal disc diameter greater than perhaps 25 mm) reliably contained gonads. Anemones of this size were not common on the shroe, and some samples contained only one or two sexual individuals. No annual cycle was obvious from histological sections. Large and small oocytes were found at all times of year (Fig. 10.78). The largest oocytes were some 240 μm in diameter, with nuclei

up to 60 μm across. At all sizes, the oocytes broadly resembled those of *A. fragacea*.

The cytoplasm of large oocytes contains fibrillar granules, compound yolk granules, cortical granules and lipid droplets (Fig. 10.80), all similar to those of *A. fragacea*. There is some dimorphism among the compound yolk granules, some containing few, large lipidic inclusions, while others contained numerous small ones. The distinction is less marked than in *Tealia*, however, and intermediate forms were found.

Cortical granules and low density granules occur in the peripheral ooplasm, and the oocytes bear tufts of cytospines (Fig.. 10.81). Amoebocytes were found among the cytospines, adjacent to the basal lamina (Fig. 10.82) just as in *A. fragacea*. The trophonema also seems similar. During the summer months, large oocytes undergoing fragmentation into membrane-bound packets were commonly observed (Fig. 10.81).

The gonads are usually pale pink in colour, and the gonad epithelial cells contained very few algal cells (Figs. 10.78, 10.79), although these were often numerous in the rest of the mesentery.

DISCUSSION

The finding of oocytes of all sizes throughout the year in *Cereus*, *Tealia* and *Anemonia* does not necessarily indicate that these species do not have an annual gametogenic cycle. If occyte growth and maturation takes considerably longer than one year, as it does in some other anthozoans (Fadlallah and Pearse, 1982a), and is only weakly synchronized, as in *A. fragacea*, then the cycle may be obscured in histological

studies. *Cereus* individuals, when maintained in the laboratory, produce juveniles steadily throughout the summer months. *Cereus* is thought to reproduce parthenogenetically, at least under some circumstances (Rossi, 1974). The scarcity of male gonads might argue for this mode of reproduction in the population studied here. The combination of brooding and parthenogenesis might favour multiple or continuous spawning, rather than single, massive spawnings, and hence the need for synchronized occyte maturation may be reduced. In *Tealia* and *Anemonia*, the sexes appear to be separate, with approximately equal numbers of males and females. These species do not brood, but spawning was not observed and little is known about their development.

The earliest female germ cells observed in *Cereus* tended to be clustered in groups, and several clear instances of mitotic activity were found among these cells. In *A. fragacea*, the corresponding cells tended to be randomly scattered, and no clear evidence of mitotic activity was obtained. In view of the considerably greater number of cells examined in *A. fragacea*, it seems likely that this apparent absence of mitosis does reflect a significant difference between the two species. It may be that an oogonial proliferative phase is simply absent during *A. fragacea* oogenesis, but present in *Cereus*. Another possibility is that this phase exists in *A. fragacea* but is very restricted in time or space and so was missed in sampling. The generally poorer synchronization of oocyte development in *Cereus* might mean that the oogonial phase continues over a much longer period, and so was encountered more frequently during the sampling procedure. The cellular origin of the germ cells is still unclear in

both species, however.

The occurrence of an oogonial phase in *Cereus* does not necessarily affect or invalidate the argument given in Chapter 4, that anemone germ cells may become oocytes at a small size and prior to their entry into the mesogloea. No signs of mitotic activity were found in cells larger than 8 μm diameter in *Cereus* or *A. fragacea*, and synaptinemal complexes were found in cells of this size. In *Cereus*, as in *A.fragacea*, the germ cells can apparently enter the mesogloea at a range of sizes, and presumably some must enter at a very small size in order to be found engulfed by larger mesogloeal oocytes. However, there is no evidence that cells as large as 20-40 μm undergo mitosis and so must still be oogonia as indicated for *Epiactis prolifera* by Dunn (1975) and *Anthopleura elegantissima* by Jennison (1979).

In *Cereus*, the concentration of ribosomes in the cytoplasm of small oocytes, and hence the electron density of their cytoplasm, appears to increase during the endodermal phase. In *A. fragacea*, they are always numerous and the cytoplasm appears dense even in the earliest stages. Neither case is consistent with the statement of Schäfer and Schmidt (1980) that ribosomes appear in the cytoplasm during entry into the mesogloea; however, *Cereus* oocytes come closer to this situation than those of *A. fragacea*.

Most of the granules and inclusions which accumulate during vitellogenesis in *Cereus* appear to be directly comparable to those of *A. fragacea*. One type of inclusion with apparently no counterpart in *A. fragacea* were the irregular, coarsely granular bodies. These resemble the primary yolk material described by Schafer (1983). However, they were only found in

small numbers, and no evidence of their conversion into other granule types was obtained.

Fibrillar granules were found in vitellogenic oocytes of all the species studied with the exception of *A. equina*. Schäfer and Schmidt (1980) suggested that fibrillar granules were involved in the formation of the fibrillar cores of the cytospines. Since *Cereus* oocytes contain abundant fibrillar granules, but have small microvilli with poorly developed cores, while *A. equina* oocytes have well-developed cytospines but no fibrillar granules, their suggestion seems unlikely. In *Cereus*, Golgi complexes appear to give rise to granules containing finely granular material, within which fibrillar areas appear at a later stage. Loosely packed fibrillar granules were rarely encountered in this species.

In *Cereus*, the compound yolk granules displayed a range of forms with different sized lipidic inclusions. In this species vesicular granules would appear to be merely an extreme form of compound yolk granule, with very small inclusions, rather than a separate granule type. The dense matrix component of some granules showed a paracrystalline substructure, similar to that described by Schäfer (1983), but which was never observed in *A. fragacea*.

In *Cereus*, as in *A. fragacea*, rough endoplasmic reticulum was often found wrapped closely around lipid droplets. The very large lipid droplets found in *Tealia* oocytes, however, appear to arise without any ER involvement. The association between lipid droplets and smooth ER described by Schäfer (1983) was not observed in any of the species studied here. The eggs of *Tealia crassicornis* are of similar size to those of

Tealia felina, and also float in seawater (Chia and Spaulding, 1972). The lipid droplets in this species are only some 5 µm in diameter. Wedl and Dunn (1983) working with *Urticina lofotensis* (which may be synonomous with *T.crassicornis*) found lipid droplets 10-15 µm across, similar to those in *T. felina*.

Low density granules close to the oolemma were observed much less freqeuntly in *A. equina* than *A. fragacea*. This may be related to the absence of fibrillar granules in *A. equina* oocytes. However, the question as to whether low density granules arise from fibrillar granules or from cortical granules remains uncertain.

The uptake of small cells by anthozoan oocytes has been reported previously. Schäfer (1981) reported such a process at the light microscope level for *Cereus*, and Schmidt and Schäfer (1980) observed it with the electron microscope for the Edwardsiid *Alfredus lucifugus*. The latter authors suggest that the ingested cells are oocytes, but also state that they originate in the trophonema, and that trophonemal cells themselves are taken up by the large oocytes. The present study points to the ingested cells being oogonia or small oocytes, but produced no evidence that they are taken up in the region of the trophonema. In *A. fragacea*, where trophonema formation was examined in some detail, it appears that the trophonemal cells are derived from epithelial rather than germ cells (see Chapter 5). In *Cereus*, it appears that the ingested cells are early germ cells which enter the mesogloea at an early stage, contact a much larger oocyte and are phagocytosed by it. Some may be degenerate prior to ingestion, possibly corresponding to the Type III cells of *A. fragacea* (see Chapter 4). In some cases, the portions of the large oocytes

engaged in uptake activity, have a very indented outline, and may be producing pseudopodia or moving through the mesogloea. The oocyte as a whole is presumably tethered by the trophonema and does not migrate. Uptake seems a controlled and specific process. Close and precise memb ane apposition is established over the entire area of contact between the cells involved at an early stage. This is not a general feature of phagocytosis in other situations, and may indicate a recognition system. Granular amoebocytes are common in the mesogloea, and often contact oocytes, but apparently are never phagocytosed. Close membrane apposition does not occur between large oocytes when they come into contact: oocyte microvilli may be important in the recognition process.

Small germ cells may be taken up by growing oocytes in *Hydra* (Zihler, 1972; Tardent, 1974) and other hydrozoans (Boelsterli, 1975). The uptake of nurse cells by oocytes has also been reported in marine sponges (Fell, 1969). In these cases, the ingested cells are present in large numbers and may make a significant contribution to the nutrition of the oocyte. In *Cereus*, however, they have only been found in small numbers, and occupy only a tiny proportion of the oocyte volume. They are not packed with glycogen or other reserve materials, and so presumably make only a very small contribution to the nutrition of the large oocyte. The significance of these ingested cells is quite unclear.

The structure of the trophonema in *A. equina*, *Tealia* and *Anemonia* seems very similar to that of *A. fragacea*. The trophonema in *Cereus*, however, seems rather less elaborate. The absence of cytospines from this region must considerably

reduce the area of contact between oocyte and endoderm, and the lack of extensive intercellular junction formation may reduce the firmness and permanence of the relationship. It would also appear to be less active functionally. The ooplasm beneath the trophonema appears much less specialized and contains fewer vesicles than in *A. fragacea*. If the mechanism suggested for *A. fragacea* is correct, and pinocytotic vesicles form largely among the bases of the cytospines, then the scarcity of such vesicles may be related to the absence of cytospines. However, pinocytosis appears to occur on a greater scale over the rest of the oocyte surface in *Cereus* than in *A. fragacea;* this may compensate for the lower levels at the trophonema. Overall, the proportion of nutrient uptake taking place via the trophonema may be lower in *Cereus*.

The process of oocyte breakdown appears to be very similar in both species of *Actinia, Cereus* and *Anemonia*. In these species, large oocytes degenerate by what appears to be an orderly and controlled fragmentation process. No instances where all the large oocytes in a gonad were undergoing fragmentation were seen apart from in *A. fragacea*. The weaker synchronization of oocyte development in *Cereus* and *Anemonia* may mean that breakdown occurs at a moderate level over a long period, with no episodes of massive breakdown. Large oocyte breakdown seems less common in *Tealia;* a small number of apparently degenerate oocytes were seen with the light microscope, but none were examined ultrastructurally and the details of the process are not known.

The algal symbionts found in *Cereus* and *Anemonia* appear to be identical, and the same as those described by Taylor (1968, 1969). Originally he termed them *Symbiodinium microadriaticum*.

In a later paper, (Taylor, 1971) he revised the classification of several dinoflagellates, taking account of the findings of Kevin *et al* (1969) and proposed that these symbionts should be known as *Gymniodinium microadriaticum*. However, most recent authors have continued to use *Symbiodinium* (e.g Colley and Trench, 1983).

While the alga appears identical in both *Cereus* and *Anemonia*, there may be differences in the nature of the host/alga relationship in the two species. The gonads of *Anemonia* are pink in colour, and contain very few algae. Taylor (1968) reported that the algae found in *Anemonia* mesenteries were all degenerate or dead. He suggested that degenerate algal cells were transferred to the mesenteries from whence they were expelled. *Cereus* gonads on the other hand, are brown in colour and contain numerous symbionts. Healthy individuals, dividing cells & degenerate cells were all found, suggesting that several stages of the algal life cycle could be completed within the gonad.

Taylor (1969) found that, in *Anemonia*, up to 60% of the carbon fixed during photosynthesis by the algae could be transferred to the host tissues, and similar results have been obtained with other marine coelenterates (e.g Muscatine and Cernichiari, 1969; Trench, 1971a, b, c; Lewis and Smith, 1971; Shick and Dykens, 1984). The host may also benefit from oxygen produced by the algae (Shick and Brown, 1977). Thus the large numbers of symbionts found in *Cereus* gonads could be of nutritional and metabolic benefit to the developing oocytes. Nutrient transfer could also take place when algal cells die and break down within host tissues. Taylor (1968) suggested that this transfer did not occur in *Anemonia* mesenteries,

since the outer layer of the algal cell could not be digested by the host and presented a barrier to any nutrient transfer. In *Cereus* gonads, during the present study, however, algal cells were observed in apparently advanced stages of degeneration and in many cases their outer layers seemed to have disintegrated. It thus seems possible that, in this species, nutrient transfer from degenerate alga to host could take place.

No algal cells were observed inside or closely associated with oocytes at any stage in *Cereus* or *Anemonia*, so it seems unlikely that they are transferred to the next generation via the oocyte. Brooded *Cereus* juveniles contain zooxanthellae, which they may take up while within their parent's gastrovascular cavity. How algae are transferred in the non-brooding *Anemonia* is unclear.

Schmidt and Schäfer (1980; Schäfer and Schmidt, 1980; Schäfer, 1983) have examined oocytes from over 40 anthozoan species, withaa view to using features of oocyte structure as indicators of taxonomic and evolutionary relationships within the group. However, a range of reproductive strategies was found even among the small number of anemone species studied here. Reproductive tactics such as brooding, parthenogenesis, hermaphroditism, and the formation of large, floating eggs may all require modifications of the oocyte structure. Until the overall strategies of individual species have been studied in some depth, the significance of particular features of oocyte morphology will always be uncertain.

Chapter 11

THE ESTABLISHMENT OF THE TESTICULAR CYSTS

INTRODUCTION

In sea anemones generally, spermatogenesis takes place in numerous testicular cysts which are located in the mesogloeal layer of the gonad (Dewel and Clark, 1972). However, as in the female, the germ cells arise initially among the basal regions of the gonad epithelial cells, and migrate into the mesogloea and establish the testicular cysts.

Early male anthozoan germ cells are small and are difficult to distinguish by light microscopy prior to their entry into the mesogloea. Thus most light microscope studies of anthozoan spermatogenesis give few details of the endodermal phase of germ cell development (Chia and Spaulding, 1972; Chia and Crawford 1973; Dunn, 1975; Jennison, 1979; Rinkevich and Loya, 1979; Szmant-Froelich *et al*, 1980). Chia and Rostron (1970) described gametogenesis in *Actinia equina*, but made no mention of a pre-mesogloeal phase. The limited number of electron microscope studies of anthozoan spermatogenesis have tended to deal mainly with mature sperm structure and spermatelliosis rather than the earlier stages (Dewel and Clark, 1972; Clark and Dewel, 1974; Hinsch and Clark, 1973; Hinsch, 1974; Lyke and Robson, 1975; Kleve and Clark, 1976; Schmidt and Zissler, 1979; Larkman and Carter, 1980; Schmidt and Holtken, 1980; West, 1980). This chapter describes the early male germ cells while within the endoderm, their entry into the mesogloea and the establishment of the testicular cysts. The process of sperm differentiation will be described in the next chapter.

RESULTS

In *A. fragacea*, the gonads appear to persist from one breeding season to the next. Spawning occurs in summer, during June and July, and the next season's male germ cells do not appear usually until September, so there is a short period when the male gonds seem to be devoid of germ cells.

The germ cells arise among the bases of the gonadal epithelial cells. Initially they occur singly, but by October they are found mainly in small groups, and soon begin to migrate into the mesogloea of the gonad. Some individual cells may enter the mesogloea singly, but most enter as groups or 'slugs' of several cells. Once within the mesogloea, the groups of germ cells enlarge rapidly by mitosis and possibly also by fusion with other groups and single cells to form the testicular cysts. By January, the cysts may be 50 μm in diameter, and the cells at the centres of the cysts begin to undergo meiosis and differentiate into spermatocytes, spermatids and eventually sperm. The major events in the establishment of the testicular cysts are summarized in Diagram 18. Throughout this chapter, the early male germ cells prior to the completion of entry into the mesogloea are referred to as prospermatogonia. The following description of the prospermatogonia applies both to single cells and those found in groups since their appearance and organelle complement are similar.

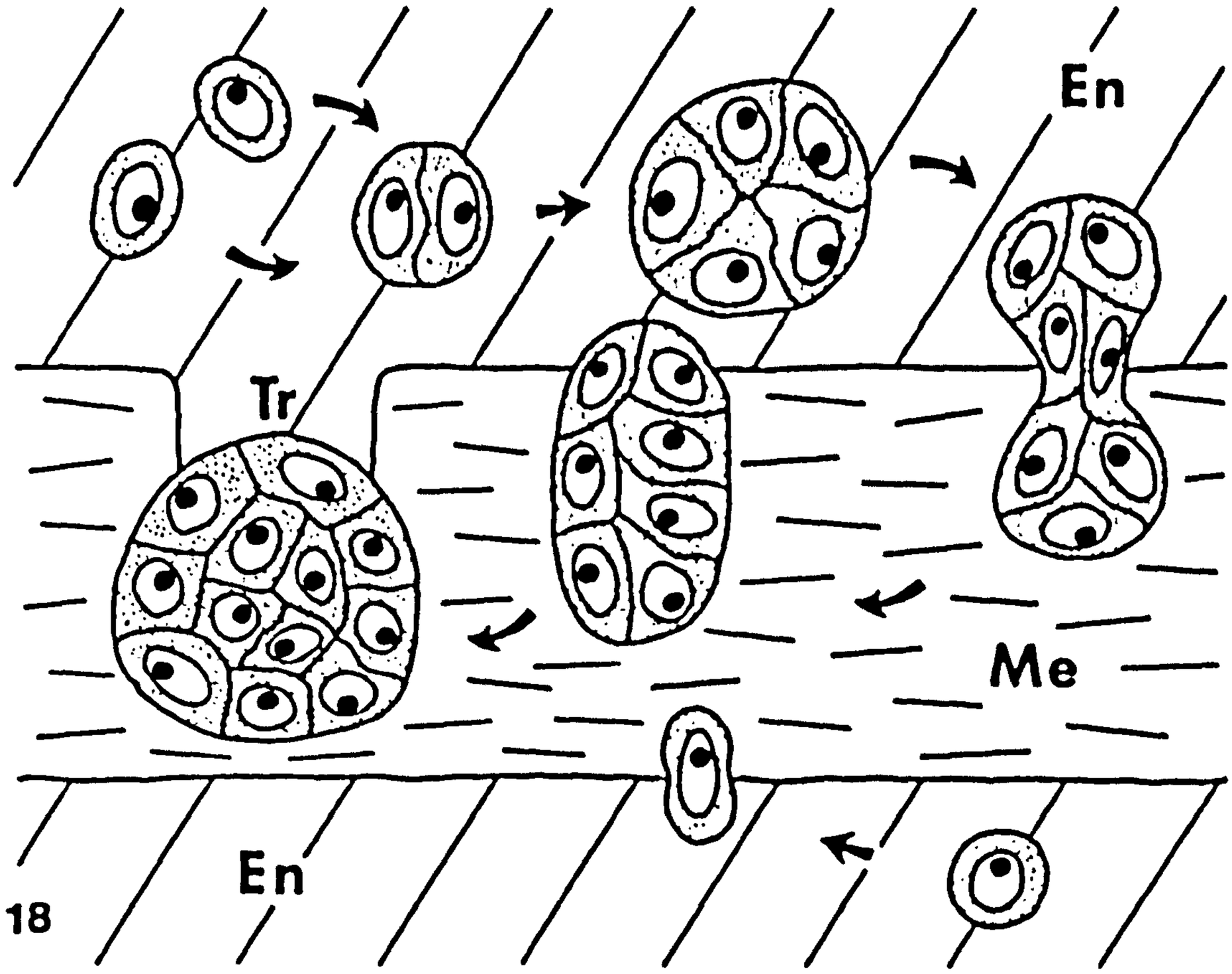


Diagram 18.

Summary diagram showing the major steps in the establishment of a testicular cyst. Prospermatogonia arise singly in the endodermal epithelium (top left), and form groups by aggregation or mitosis or both. These groups then migrate into the mesogloea (centre right), but the last part of the group to enter retains contact with the endoderm and a trophonema develops (centre left). A small proportion of prospermatogonia (bottom right) enter the mesogloea singly.

1. Structure of Prospermatogonia

The prospermatogonia are usually first seen lying a few micrometres away from the gonadal mesogloea, among the tangled bases of the endodermal epithelial cells (Fig. 11.1). They are small cells, usually 6-7 μm in diameter and usually spherical or slightly elongate. They have relatively large nuclei and densely staining cytoplasm. The nucleus is usually 2.5-4 μm in diameter and is of moderate electron density with irregular patches of more dense chromatin material. It also contains numerous small dense granules, 50-70 nm in diameter (Fig. 11.2). There is usually a single nucleolus which is always located at the periphery of the nucleus, against the nuclear envelope. Rarely two nuclei may be found. The nucleus is surrounded by a typical double membrane nuclear envelope, with occasional nuclear pores. The outer membrane of the envelope is usually studded with ribosomes.

The cytoplasm of the prospermatogonia is generally dense compared with that of the surrounding epithelial cell bases, and contains numerous free ribosomes. The cytoplasm contains a small amount of rough endoplasmic reticulum, which may be present as short lengths, but typically is arranged as individual long cisternae (Fig. 11.2) which may extend for several micrometres around the cell, often close to the surface. The cisternae are narrow and contain material of moderate electron density.

The mitochondria usually appear squat and average about 0.3 x 0.6 μm in section, with a dense matrix and numerous narrow shelf-like cristae (Fig. 11.3). They are usually clustered into groups rather than randomly scattered through the cytoplasm.

Prospermatogonia are usually seen to contain a single Golgi complex, consisting of a few flattened parallel cisternae (Fig. 11.3). The ends of the cisternae may be dilated and contain moderately electron dense material, while the complex is often surrounded by small, membrane-bound vesicles, 40-80 nm in diameter, containing a similar material.

The cytoplasm contains dense membrane-bound vesicles of various kinds. The largest may be 0.75 μm or more in diameter, are spherical and contain a highly electron dense homogeneous material (Fig. 11.1). Commonly they are rather smaller, less regular in shape and have heterogeneous contents which may include membranous material (Figs. 11.4, 11.5). These smaller bodies are often found in clusters. Some bodies have loosely fitting membranes, giving them a 'haloed' appearance. Some dense bodies contain an angular electron lucent core (Fig. 11.4). Similar bodies have been found in many coelenterate cell types. They may show acid phosphatase activity and are often referred to as residual body-like inclusions (Chapman, 1974; Tiffon and Hugon, 1977; Larkman and Carter, 1980). Multivesicular bodies, which are of lower electron density and contain numerous small vesicles are commonly found (Fig. 11.6). Small apparently empty membrane-bound vesicles are also common (Fig. 11.4).

Glycogen is present in all prospermatogonia, usually in the form of numerous small deposits scattered throughout the cytoplasm (Fig. 11.2), although larger deposits are occasionally found. Lipid droplets are also common. These may be 1 μm in diameter, and often display areas of lower density, which may indicate extraction of components during tissue processing (Figs. 11.3, 11.5).

Each prospermatogonium appears to possess a single flagellum. Occasionally the flagellum arises at the surface of the cell, but more commonly it originates some distance from the surface and runs in a groove or channel in the cytoplasm. The groove may be shallow and open (Fig. 11.1), deeper and virtually sealed over (Fig. 11.3) or, most commonly, it may be a tube, sealed along its length, but open at one end (Figs. 11.4, 11.7). It is not clear how far the flagellum projects beyond the surface of the cell. The flagellum originates from a basal apparatus consisting of two centrioles at right angles to each other. Occasionally a third or accessory centriole is seen (Fig. 11.8), and the basal apparatus may be seen to include a striated rootlet (Fig. 11.9), forming a basal-body-rootlet complex similar to that found in many coelenterate cells (see Chapter 3). The flagellum has a typical '9+2' arrangement of microtubules for most of its length. Near its insertion, it shows the usual elaborations which have been described for other coelenterate flagella and sperm tails (Dewel and Clark, 1972; Larkman and Carter, 1980), including a region where Y-shaped fibrils join the microtubular doublets to the flagellar membrane (Fig. 11.11). Lengths of flagellar axoneme are frequently found free in the cytoplasm, not enclosed by a plasma membrane (Figs. 11.10, 11.11). These cytoplasmic axonemes usually have a precisely organized '9+2' arrangement, but occasionally disrupted patterns are seen (Fig. 11.12), the significance of which is not known.

The prospermatogonia contain both dense and fibrillar nuage materials (Figs. 11.13, 11.14) which are identical to those found in early oocytes (see Chapters 4 and 5). As in the female, more than one area of nuage may occur within a

single cell. Hooped fibrillar structures (Fig. 11.15) similar to those found in oocytes (see Chapter 6, Section G) are also found, although less commonly. Particulate nuage has not been observed in prospermatogonia, although it does occur in later stages (see Chapter 12).

Most of the prospermatogonia found singly in the endoderm are smooth in outline, but some display slender cytoplasmic processes, which may extend towards and make contact with the mesogloea. Occasionally, small side branches extend out from the main gonadal mesogloeal layer and may contact individual prospermatogonia. Both situations are seen to occur in Fig. 11.16. Some prospermatogonia appear to have a deep, cylindrical invagination of the cell surface which may contain small vesicles and may be lined with a basal lamina-like material (Figs. 11.1, 11.11).

The prospermatogonia appear to arise singly, but by October or November, most are found as pairs (Fig. 11.17) or small groups (Fig. 11.18). Usually the cells comprising these groups are closely packed, being separated from each other by a narrow and apparently empty intermembrane gap. Rarely, desmosome-like intercellular junctions are found between adjacent cells (Fig. 11.22). Not uncommonly, however, the cells within a group are separated by slender processes from the surrounding endodermal epithelial cells (Figs 11.13, 11.17). These processes often contain bundles of microfilaments (Fig. 11.13) or microtubules (Fig. 11.21). Occasionally the space between neighbouring cells contains undulating membranous sheets or whorls (Figs. 11.19, 11.20). This is most pronounced around a small minority of cells which appear to have shrunk away from their neighbours (Fig. 11.19).

In section these 'shrunken cells' appear to have only a very thin rim of cytoplasm surrounding the nucleus.

Intercellular bridges have not been observed between the prospermatogonia comprising the groups within the endoderm. In some groups, the cells appear to radiate out from a central focus (Fig. 11.18), and often pairs of cells are found separated by a mass of small membranous vesicles (Fig. 11.20), but cytoplasmic continuity has bever been observed. No instances of cells undergoing mitosis while within endodermal groups have been seen, but this should not be taken as strong evidence that mitosis does not occur (see Discussion).

In the region where the gonad joins the mesenteric retractor muscle, the epithelial cell muscle processes are more highly developed and the mesogloea is thicker and contains more collagen fibrils which are more regularly packed than is normal in the rest of the gonad. Cells which in many ways resemble germ cells are frequently encountered in this region. These cells tend to be elongate and spindle-shaped rather than rounded, and their mitochondria tend to be small and dense, but otherwise they are very similar to the prospermatogonia described above. They lie in the usual position, a few micrometres back from the mesogloea and the muscle processes (Fig. 11.23). Whether their unusual elongate shape is merely imposed by shape changes in the muscular tissue around them, or whether they represent a separate cell population, distinct from the majority of male germ cells, is not known.

2. Entry into the Mesogloea

After a period within the endodermal cell layers, the prospermatogonia migrate into the mesogloea of the gonad. This process was observed in samples taken during October and November. A small proportion of cells may enter the mesogloea singly (Fig. 11.24) but by this time most prospermatogonia occur in the endoderm as groups, and these groups enter the mesogloea as 'slugs' or elongate clusters of cells. The mechanism of locomotion of both 'slugs' and single cells is not clear. The cells show no obvious morphological specializations for movement, but move between the epithelial cell bases by what is presumed to be some form of amoebold movement, as was suggested for oocytes (see Chapter 5). The epithelial cell bases themselves may contribute to the movement of the germ cells. By whatever means, the prospermatogonia separate the bases and muscle processes and make contact with the basal lamina lining the mesogloea. Once contact has been made, mesogloeal outgrowth around the germ cells may play an important part in the entry process. In Fig. 11.25, a group of prospermatogonia lies in contact with the basal lamina, but as yet does not protrude beyond the line of the mesogloeal-endoderm boundary. Slender extensions of the mesogloea appear to be growing out around the edges of the group of germ cells.

Soon, however, the group of prospermatogonia does protrude into the mesogloea proper. As the cells pass into the mesogloea, they do not break through the basal lamina but rather push it into the mesogloea ahead of them. Thus the 'slug' of prospermatogonia becomes covered by the basal lamina as it enters (Fig. 11.26 and Diagram 19). During entry, the 'slug'

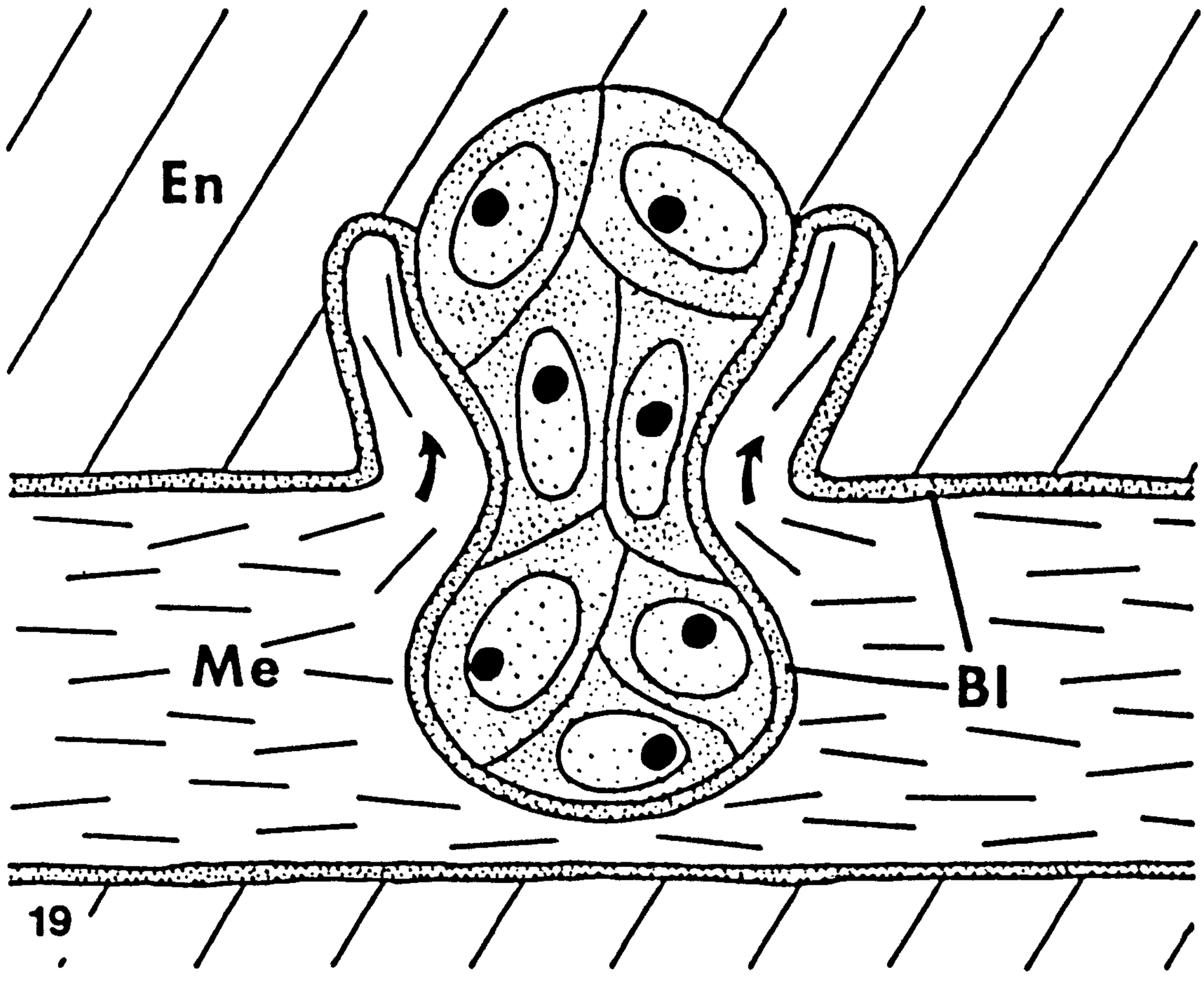


Diagram 19.

Diagram illustrating the main features of entry into the mesogloea. The group or 'slug' of prospermatogonia becomes constricted into an hourglass shape as it passes from the endoderm into the mesogloea. Mesogloeal outgrowth (arrows) usually occurs around the entering group, and the group becomes covered by the basal lamina during entry.

is usually constricted at the mesogloea/endoderm boundary, giving it a distinctive hour-glass shape (Fig. 11.28). Some of the cells within such a 'slug' become elongate during this process, but whether this shape change is active or passive is not known. The leading part of the group expands within the mesogloea as more cells enter, finally producing a roughly spherical group of cells within the mesogloea after entry is complete.

It appears that the last part of the prospermatogonial group to enter the mesogloea retains contact with the endodermal cell bases adjacent to it, and these bases are dragged into the mesogloea behind the germ cells. This produces a 'plug' of endodermal bases between the mesogloeal outgrowths around the prospermatogonia (Fig. 11.27). This 'plug' prevents overgrowth of this region by the mesogloea, and the contact between germ cells and endoderm is maintained throughout spermatogenesis. It also means that the basal lamina around the germ cells remains continuous with that of the gonadal epithelium. Close membrane contact between endoderm and germ cells occurs in this region, although no elaborations such as intercellular junctions have been observed forming between the two. The endodermal cell bases are often rich in glycogen and lipid droplets, and may contain bundles of microfilaments. At a later stage, the endodermal cells in contact with the germ cells begin to specialize and give rise to a structure which, by analogy with the female gonad, may be termed a trophonema (see Discussion). The nuclei of the endodermal cells are normally located apically but after the initiation of the trophonema, endodermal nuclei are often

found close to the region of contact with the germ cells (Fig. 11.29). The major features of this stage are shown in Diagram 20.

During and soon after entry into the mesogloea, many groups of prospermatogonia contain usually a single cell of rather different appearance. These cells are the same size and shape as the germ cells, but are of much lower electron density, and hence have been termed 'light cells' (Fig. 11.30). Their nuclei are surrounded by an intact nuclear envelope but contain only a faint, finely fibrillar material. Their cytoplasm also appears sparse and poorly organized (Fig. 11.31). It may contain normal-looking mitochondria, but other organelles are either disrupted or absent. The cell membrane may also appear discontinuous. The cytoplasm may contain axonemal microtubules and structures resembling nuage material, so it seems likely that these cells originate from germ cells, although they now appear to be degenerate. Occasionally, one or more prospermatogonia may appear to become separated from a group and be 'left behind' in the endoderm as the group enters the mesogloea. These cells appear to degenerate rapidly (Fig. 11.27) but at no stage come to resemble 'light cells'.

As the groups of germ cells enter the mesogloea, they very often become associated with granular amoebocytes. The amoebocytes become flattened around the groups of prospermatogonia and are always positioned in a similar way, which suggests that this association is not merely fortuitous. They come to lie closely against the layer of basal lamina which surrounds the germ cells, and thus separate the basal lamina from the fibrous mesogloea (Fig. 11.32). A variable

20 Gv

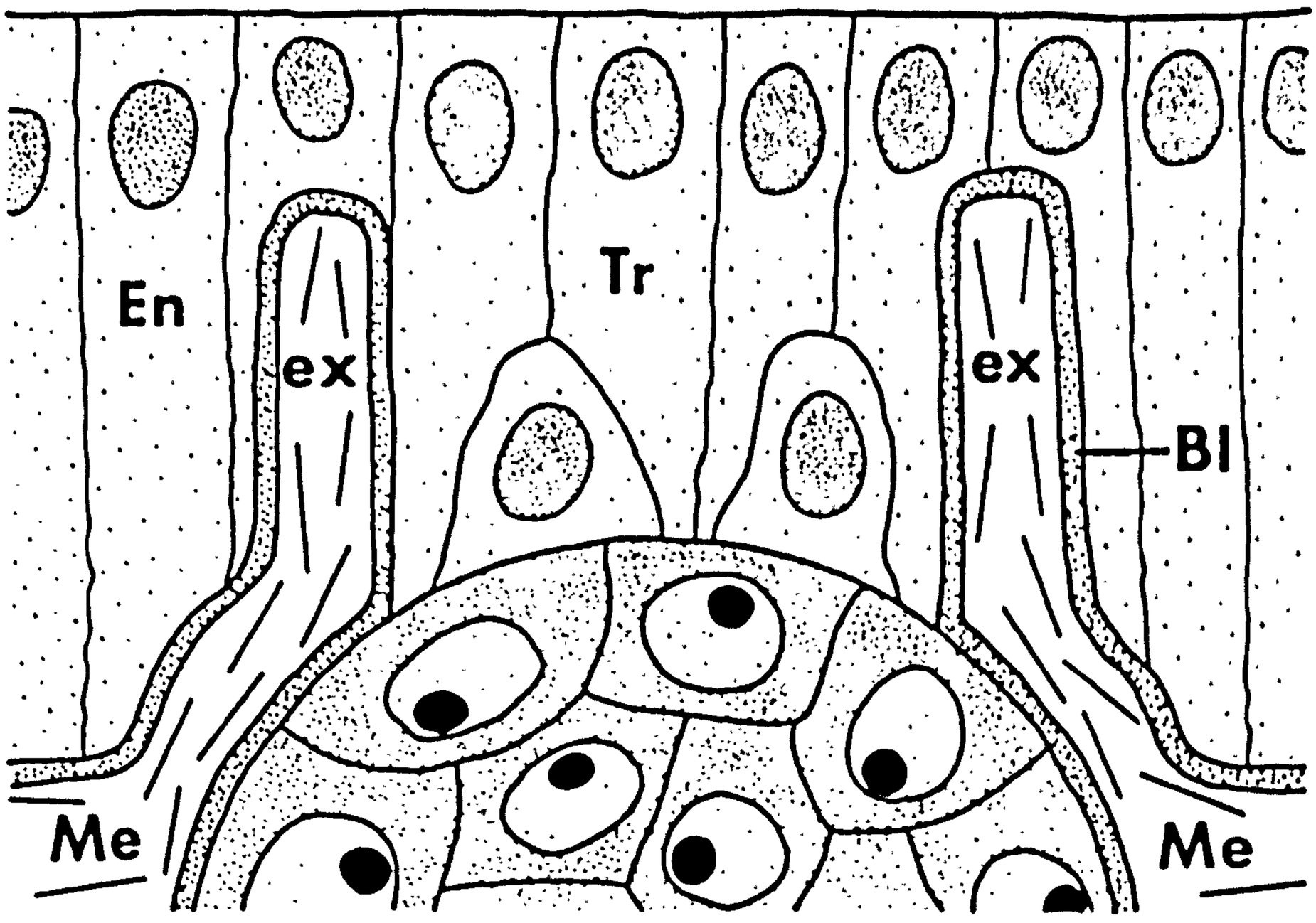


Diagram 20.

Diagram illustrating the relationship between the spermatogonia (bottom) and the endoderm soon after entry into the mesogloea. The endodermal epithelial cells between the mesogloeal extensions (ex) remain in contact with the spermatogonia and begin to specialize to form the trophonema. Some of these cells appear to depart from the normal epithelial arrangement and have their nuclei close to the interface with the germ cells.

proportion of the surface of the prospermatogonial group may become covered in this way, but amoebocytes never form a continuous layer around the germ cells.

Once entry into the mesogloea is complete, the groups of germ cells enlarge to form the testicular cysts in which sperm differentiation takes place. The original gonad mesogloea was only a few micrometeres thick, and the testicular cysts may reach a diameter of 150 μm at maturity, so the mesogloea and epithelial sheets of the gonad are deformed as the cysts enlarge. The layer of mesogloea around the cysts becomes thinner and less well endowed with collagen fibrils as the cysts grow (Figs. 11.33-11.35). Cells undergoing mitosis are seen occasionally, although not frequently, in early growing cysts (Fig. 11.34). In these cells, the mitochondria and cisternae of endoplasmic reticulum may become ringed around the nuclear area as mitosis proceeds. The cysts may reach a considerable size, perhaps 50 μm in diameter (Fig. 11.35), before cells at the centre of the cysts begin to differentiate to become spermatocytes and begin meiosis.

During January and February, at a time when most prospermatogonia have left the endoderm and entered the mesogloea, cells resembling germ cells are occasionally found in the endoderm. These cells are often found at the periphery of the gonad, especially near its junction with the retractor muscle. These cells may be larger than most prospermatogonia, reaching 10 μm in diameter. Their cytoplasm may be more extensive and contain greater quantities of glycogen and lipid (Fig. 11.36). It may also contain nuage material and hooped fibrillar structures. Thus these cells closely

resemble prospermatogonia or perhaps oocytes of similar size.

DISCUSSION

The early male germ cells of *A. fragacea* described in this thesis are similar in appearance, although not in behaviour, to the early female germ cells described in Chapters 4 and 5. Both types of cell morphologically resemble the interstitial cells found in Hydrozoa. These have been extensively studied, especially in *Hydra* (for a review, see Bode and David, 1978) and which are known to give rise to the gametes. However, the origin of anthozoan germ cells is less certain, and the present study has not provided evidence to confirm previous light microscope reports (Dunn, 1975; Jennison, 1979; Szmant-Froehlich *et al*, 1980) that anthozoan germ cells also derive from interstitial cells. In *A. fragacea* the prospermatogonia seem to arise, evenly distributed throughout the gonad, in early autumn. No population of interstitial cells could be identified within the gonad prior to that time. It is possible that the germ cells could arise at an extra-gonadal site and migrate into the gonad, but no evidence of this was found. At present, the origin of the germ cells of both sexes in *A. fragacea* remains unclear, and in the absence of firm evidence, it is perhaps unwise to assume that they derive from interstitial cells. It should perhaps be mentioned that, in a recent review, Miller (1983) quotes Schincariol and Habowsky (1972) as indicating that the gonads in *Hydra* arise by the de-differentiation of ectodermal epithelial cells into cells which form the spermatogonia. However, there is no mention of such a process in this paper

and it is not the generally accepted mechanism.

Schmidt and Holtken (1980), speaking of the Anthozoa as a whole, state that the early male cells differ from early oogenic cells only by having a prominent centriolar complex. In *A. fragacea*, however, this distinction would not appear to hold true, since the early female cells may have flagella and centriolar complexes identical to those found in the male. The present study has revealed minor differences between early male and female germ cells, although they are undoubtedly very similar. In the population of anemones studied here, the early female germ cells first appear in the endoderm in late spring and enter the mesogloea throughout the summer months. Prospermatogonia, on the other hand, first appear in September or October, and generally have entered the mesogloea by December. The prospermatogonia tend to have more dense nuclei with better-defined nucleoli than do female germ cells of comparable size, and their cytoplasm contains more dense bodies and multivesicular bodies than in the female. The single long cisternae of endoplasmic reticulum would also seem to be a predominantly male characteristic, and the male cells usually contain less glycogen and lipid than female cells of the same size. However, these differences are not marked, and may be obscured by the considerable variation in appearance shown by different cells even from the same animal.

In *A. fragacea*, flagella and associated centriolar structures were found even in the very earliest male germ cells observed. Flagella were found among groups of prospermatogonia in the endoderm and entering the mesogloea which did not appear to contain any later stage cells. Dewel and Clark (1972) found flagella and associated structures in sperm-

atocytes of the sea anemone *Bunodosoma cavernata* and remarked that it was interesting that they should occur in pre-spermatid stages. Hanisch (1972) found that in the hydrozoan *Eudendrium racemosum* flagella first appear at the primary spermatocyte stage. Schmidt and Holtken (1980) found flagella in early anthozoan male cells, and raised the possibility that this finding might be evidence that the germ cells had arisen from flagellated endodermal epithelial cells. While no evidence that this was the case was obtained in the present study, it remains an interesting suggestion.

Schmidt and Holtken (1980) also found flagellar axonemes lying within the cytoplasm of anthozoan germ cells, as was found in *A. fragacea*. They suggest that they might represent a stage in the formation of the typical external flagellum. Such cytoplasmic axonemes were not found in early female germ cells in *A. fragacea* and have not been reported from females of other anemone species.

The finding of nuage material in male as well as female germ cells increases the likelihood of it having a specific role in germ cell determination, rather than it being some form of metabolic intermediate as has been suggested in other species (e.g Kessel, 1968a; Boelsterli, 1977). The possible significance of nuage has been discussed in Chapter 6, Section G.

The prospermatogonia generally form goups within the endoderm before they enter the mesogloea. How these groups arise is not clear. It seems likely that they arise by mitosis of individual prospermatogonia, but confluence of individual cells to form groups may well also occur. Mitosis was not observed among endodermal prospermatogonia, but then

it was only rarely observed in growing testicular cysts in the mesogloea, where presumably mitosis must occur frequently. It has been suggested that mitosis may be of short duration in sea anemones (Doumenc, 1977) and so is rarely seen in random sections. In contrast, Schincariol and Habowsky (1972) were able to describe mitosis in the primary spermatogonia of *Hydra fusca* in some detail. In some endodermal goups in *A. fragacea*, the prospermatogonia are closely packed and separated by only narrow intercellular gaps. In other groups, however, slender processes from the surrounding epithelial cells extend between the germ cells. This might suggest that the cells in these groups had migrated towards each other rather than forming as the result of mitosis. Alternatively, the epithelial processes might push their way between the daughter cells after mitosis. Many of the processes contain bundles of microfilaments and microtubules, and so could be capable of such movements.

The spermatogonia of many animal species including mammals tend to be connected by intercellular bridges (Dym and Fawcett, 1971). Such bridges have also been reported between *Hydra* spermatogonia (Stagni and Lucchi, 1970). These bridges are the result of incomplete cytokinesis after cell division, and may serve to synchronize the development of conjoined cells. In *A. fragacea*, intercellular bridges occur between spermatids (see Chapter 12), but have not been observed between spermatogonia. Several prospermatogonia within a group sometimes appear to radiate from a common focus, and pairs of cells may give the impression of having only recently separated, but cytoplasmic continuity between prospermatogonia has not

been observed. The significance of the vesicles and undulating membranes found between some cells in groups is not understood. Thus, from the present study, it is not possible to ascertain whether the groups of cells which migrate into the mesogloea are 'clones' arising from the division of a single early germ cell, or are aggregations of cells from different 'clones'.

After the endodermal phase, the groups of prospermatogonia enter the mesogloea, although a small proportion of cells may enter singly. The migration of groups of male germ cells into the mesogloea has been reported for the anemone *Anthopleura elegantissima* by Jennison (1979). The method of movement of these 'slugs' of cells is not clear, but their behaviour is similar in many ways to that of oocytes which enter the mesogloea of the female gonad individually. In both cases, the cells display no obvious specializations either for cell locomotion or for penetrating the mesogloea. Slugs of prospermatogonia often become constricted into hour-glass shapes as they enter, a situation often encountered during oocyte entry (Dunn, 1975 and see Chapter 5). Mesogloeal outgrowth around the entering cells appears to play a significant part in the entry process in both male and female, although the mechanism of this observed outgrowth is unknown. Both male 'slugs' and oocytes become covered by the basal lamina as they enter the mesogloea. Presumably extra basal lamina must be added as the cells progressively enter and then enlarge within the mesogloea, but in both cases the source of this material is uncertain.

Different authors have used various terms for the mesogloeal groups of male germ cells. Dunn (1975) described

spermatogenesis occurring in "follicles", Jennison (1979) in "vesicles", while Dewel and Clark (1972) and West (1980) used the term "testicular cyst", as has been used in the present thesis. Chia and Rostron (1970), working with *Aetinia equina*, described sperm developing in elongate tubules, reminiscent of the seminiferous tubules of vertebrates. In *A. fragacea*, spermatogenesis takes place within roughly spherical cysts rather than in tubules. Observations on *A. equina* collected from the same site as *A. fragacea* during the present study, indicate that in this species also sperm development takes place in spherical cysts, which closely resemble those of *A. fragacea*.

Schmidt and Holtken (1980) indicate that the cyst formed after the male germ cells have entered the mesogloea is closed and completely covered by mesogloea. This differs from the situation in the female where the mesogloea does not overgrow the last part of the oocyte to enter (Schmidt and Schäfer, 1980). In *A. fragacea*, however, the situation in the male appears to correspond very closely to that in the female (see Chapter 5). In female *A. fragacea*, the last part of the oocyte to enter the mesogloea retains close contact with the endoderm. This prevents mesogloeal overgrowth in this region, and is important in the establishment of the trophonema. The present study confirms the suggestion of Wedl and Dunn (1983), that trophonemata occur in both male and female gonads. The male trophonema appears to arise in very much the same way as in the female. The last part of the 'slug' of prospermatogonia to enter the mesogloea does not detach from the endodermal cell bases as it enters but rather drags them

into the mesogloea behind it. These endodermal cell bases prevent mesogloeal overgrowth from sealing off the developing cyst, and the endodermal cells in contact with the germ cells begin to differentiate into specialized trophonemal cells. These cells may be partially separated from the rest of the endoderm by the mesogloeal outgrowth around the 'slug' during entry, and this may be important in promoting their specialization. Soon after entry, endodermal nuclei are often found close to the region of contact with the germ cells. This suggests that the endoderm is no longer a simple columnar epithelium in this region, since most gonadal epithelial cells have apically situated nuclei, and might indicate that specialization of this region has begun. The structure of the fully-formed male trophonema will be described in Chapter 12.

In some members of the class Hydrozoa, spermatogenesis takes place in the ectodermal cell layer, among the bases of the epithelial cells. Long epithelial cell processes may traverse the developing spermary, and the germ cells often develop in direct contact with ectodermal cells in this way (Campbell, 1974; Roosen-Runge, 1977). In *A. fragacea*, most male germ cells appear to develop without direct contact with cells of any other type. Contact with the overlying epithelial cells is restricted to the localized area of the trophonema, and only a small proportion of the germ cells are involved. Occasionally, granular amoebocytes are found inside testicular cysts, where they may play a scavenging role, but they do not enter into a close association with any of the germ cells.

The occasional occurrence of cells of much lower electron density among groups of normal looking prospermatogonia has

not been remarked by previous authors. Perhaps the point should be made that the normal spermatogonia shown by Schmidt and Holtken (1980) are generally of much lower density than those found in the present study. In *A. fragacea*, many of these 'light cells' contain axonemes and structures resembling nuage material, so it seems likely that they are of germ cell origin. They usually appear degenerate, and so may represent prospermatogonia which for some reason fail to develop normally and break down. Possibly they derive from the 'shrunken cells' which are occasionally seen in prospermatogonial groups. The loss or degeneration of a proportion of the germ cells at some stage during spermatogenesis is common in a wide range of animal species (Roosen-Runge, 1977).

The finding of occasional germ cells apparently left behind in the endoderm long after the majority have entered the mesogloea is unexplained but raises some interesting possibilities. All that can be said with any certainty is that there appears to exist a small minority of germ cells whose behaviour and appearance differ from the rest. One possibility is that these cells are merely defective and fail to follow the normal developmental programme and eventually degenerate. None of this type of cell seen so far has shown any signs of degeneracy, however. Another possibility is that a small number of germ cells are left behind in the endoderm each year, and remain there to provide a reserve which could give rise to the following season's crop of prospermatogonia. Most of the germ cells left behind in this way are larger than the normal prospermatogonia, and they may contain considerably more reserve materials in the form of

glycogen and lipid droplets. In this respect, and in their appearance generally, they very closely resemble oocytes of comparable size (see Chapter 5). This suggests a third possibility, that these cells could initiate a female reproductive cycle on some subsequent occasion. However, studies continued from those of Carter and Funnell (1980) have provided evidence that *A. fragacea* individuals maintained in the laboratory have not changed sex during a three year period.

Chapter 12

SPERMATOGENESIS

INTRODUCTION

The structure of the mature sperm and the later stages of spermatogenesis are among the few areas of coelenterate gametogenesis which have been studied in some detail. While most publications have concerned hydrozoan species, a number of anthozoan sperm have been described at the electron microscope level (Dewel and Clark, 1972; Hinsch and Clark, 1973; Clark and Dewel, 1974; Lyke and Robson, 1975; West, 1980). Schmidt and co-workers (Schmidt and Zissler, 1979; Schmidt and Holtken, 1980) briefly examined the sperm of over 40 different anthozoan species and used features of sperm morphology as indicators of possible phylogenetic relationships. Work on coelenterate sperm and spermatogenesis has been reviewed by Hinsch (1974) and Miller (1983). The sperm of *Actinia equina* was investigated prior to the commencement of the present study, and an account of its ultrastructure has been published (Larkman and Carter, 1980). A reprint of this paper is included with this thesis.

The sperm of *Cereus pedunculatus*, *Tealia felina* and *Anemonia sulcata* were all examined by Schmidt and Zissler (1979). The sperm of *A. fragacea* appears virtually identical to that of *A. equina*, as described by Larkman and Carter (1980). The mature sperm of these species will therefore not be described here. The process of spermatogenesis appears broadly similar in all these species, and only that of *A. fragacea* will be described in any detail, while *Cereus pedunculatus* will be described briefly.

RESULTS

1. Spermatocytes and Meiosis

Typical tripartite synaptinemal complexes were found in the nuclei of many cells near the centre of developing testicular cysts (Figs. 12.1,12.2). These complexes were identical to those found in early oocytes (see Chapters 4 and 5) and strongly suggest that the cells containing them were undergoing meiotic prophase and so should be regarded as primary spermatocytes. Otherwise, however, no consistent morphological differences were observed to distinguish spermatocytes from spermatogonia.

Cells apparently in various later stages of Meiosis I are often encountered (Fig. 12.3), although the details of the different stages of meiosis are difficult to distinguish by electron microscopy. As nuclear division proceeds, two nuclear areas can often be distinguished, sometimes separated by mitochondria or cisternae of endoplasmic reticulum (Fig. 12.4). The extensive individual ER cisternae found in spermatogonia and prospermatogonia (see Chapter 11) are still present during meiosis, and may become wrapped around the nuclear areas, where they may be confused with the nuclear envelope. Sometimes, the reformation of the nuclear envelope is seen to be completed before any signs of cytokinesis can be discerned, producing a cell with two normal-seeming nuclei (Fig. 12.5). When cytokinesis does occur, it is incomplete, leaving the two daughter cells linked by a cytoplasmic bridge.

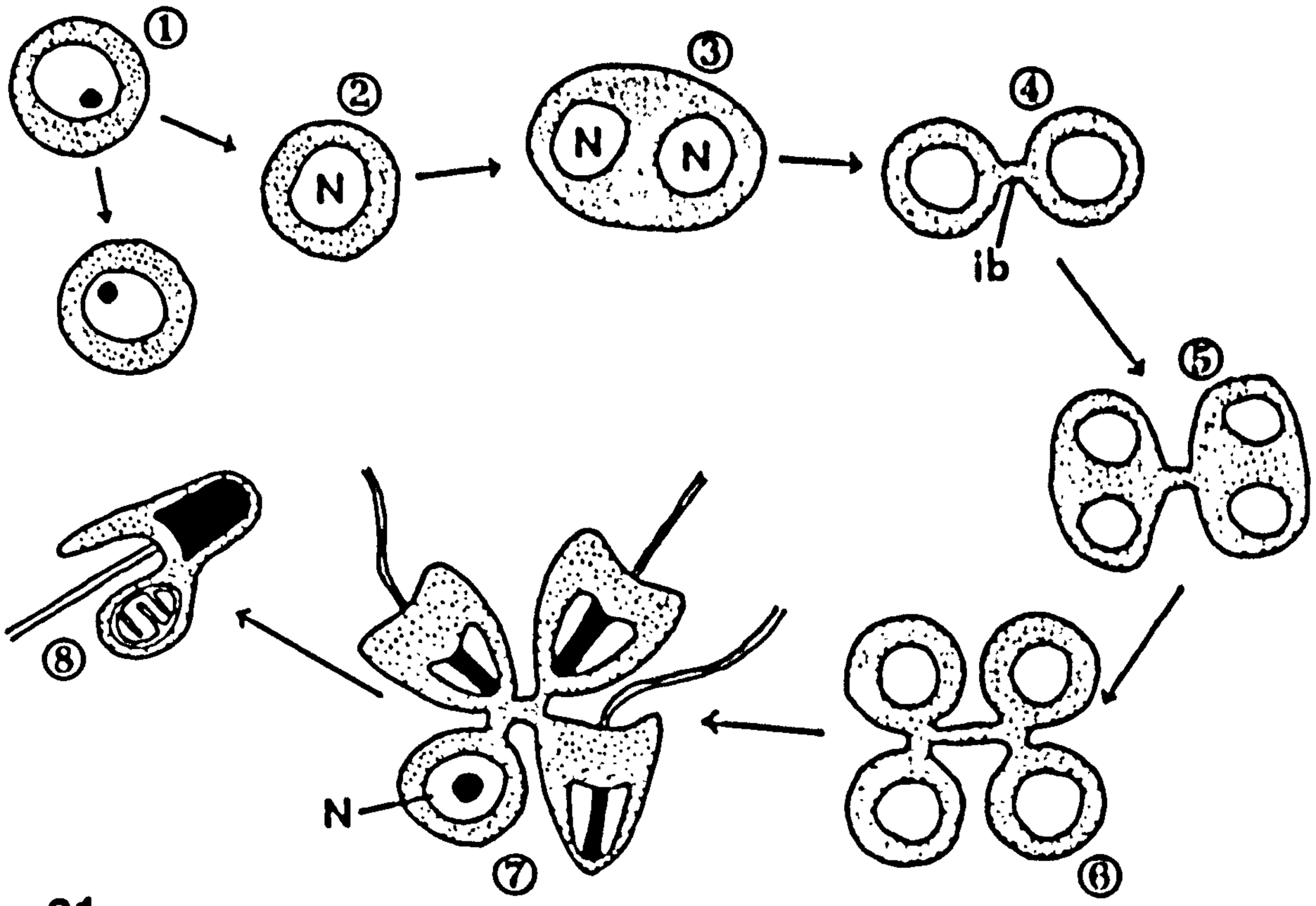
The second meiotic division appears to follow a similar pattern to the first (Fig. 12.6). Again, nuclear envelope reformation may precede cytokinesis (Figs. 12.7, 12.8).

At least in some cases, this may lead to a cell with two fully formed nuclei, linked by a cytoplasmic bridge to another cell, presumably in a similar condition (Fig. 12.9). When cytokinesis of the second meiotic division finally does take place, it gives rise to a tetrad of conjoined speratids. The cytoplasmic bridges between them are about 300 nm wide, and bear a thin layer of electron dense material on the cytoplasmic face of the plasma membrane (Fig. 12.10). The sequence of events during the meiotic divisions is summarized in Diagram 21.

2. Spermatids and Spermateliosis

The early spermatids are usually rounded cells, 3-4 μm in diameter, with relatively very large, spherical nuclei. Apart from their small size, and the presence of inter-connecting cytoplasmic bridges, they are similar in appearance and organelle complement to the earlier stages. The process of spermateliosis (also called spermiogenesis) involves a number of processes by which the apparently unspecialized early spermatid is converted into the mature spermatozoon. These principally involve modifications of the flagellum, the mitochondria and the nucleus, and are summarized in Diagram 22.

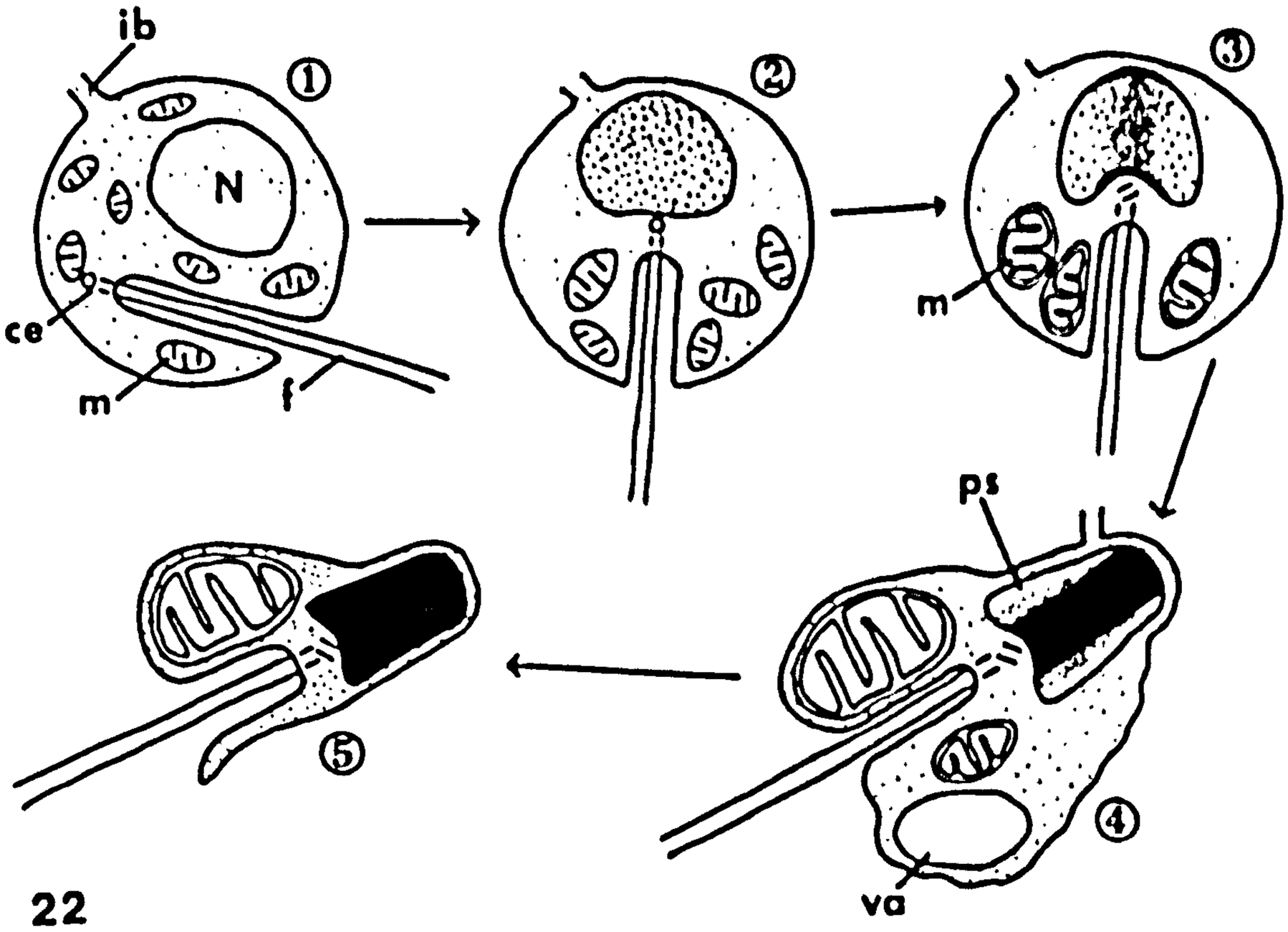
The modification of the insertion of the tail flagellum into its final form appears to take place in one of two different ways in different spermatids. This may be because individual daughter spermatids may inherit different arrange-ments from their parents after meiosis. Some early spermatids have a flagellum originating at the surface of the cell and projecting directly outwards (Fig. 12.11). In these cases, it appears that the centrioles move towards the centre of the



21

Diagram 21.

Diagram summarizing some of the main features of spermatogenesis. Spermatogonia (1) divide mitotically to produce either more spermatogonia, or primary spermatocytes (2) which then undergo meiosis. After the first meiotic division, nuclear envelope reformation may be complete before cytokinesis begins (3). Cytokinesis is incomplete, and produces a pair of secondary spermatocytes conjoined by an intercellular bridge (4). After the 2nd meiotic division, nuclear envelope reformation may precede the onset of cytokinesis (5), which is incomplete and results in 4 spermatids linked by intercellular bridges (6). The spermatids then differentiate with no preferred orientation with respect to the cytoplasmic bridges (7), which finally break to release fully-formed sperm (8).



22

Diagram 22.

Diagram illustrating some of the major processes involved in spermatid differentiation. The early spermatid (1) has numerous small mitochondria, and a finely granular nucleus. The nuclear material becomes more coarsely granular (2), mitochondrial fusion begins, and the centrioles become situated close to the nucleus. Nuclear condensation involves the formation of dense plaques (3) at the future anterior and posterior poles of the nucleus, and the accumulation of dense chromatin in the region between them. Condensation proceeds (4), to produce a dense cylinder of condensed chromatin surrounded by a peripheral space or sleeve of nucleoplasm. Mitochondrial fusion continues, while excess cytoplasm and organelles undergo breakdown in a vacuole which is later expelled. The mature sperm (5) has an almost uniformly dense nuleus, and a single mitochondrial complex located to one side of the tail flagellum.

cell and take up a position close to the nucleus. During this process, the cell membrane is invaginated around the insertion of the tail to form the cytoplasmic collar (Fig. 12.12). In other cases, the flagellar axoneme within the early spermatid runs within the cytoplasm without a surrounding membrane, as noted for some prospermatogonia (see Chapter 11). Membrane lined vesicles appear around the axoneme, initially close to the centrioles, and become wrapped around the axoneme (Fig. 12.13). These vesicles enlarge and extend distally along the axoneme until they reach the surface of the cell, resulting in a membrane-bound flagellum running in a deep invagination in the cytoplasm (Fig. 12.14). It is not clear whether the membrane-lined vesicles connect with the cell surface throughout this process, and so should be regarded as invaginations, or whether they are initially sealed.

The mitochondria accumulate at one pole of the cell, close to the insertion of the flagellum, where they increase in size and reduce in number, presumably by fusion (Figs. 12.11, 12.12). They eventually form a single mitochondrial complex, some 800 nm in diameter (Fig. 12.15). The precise arrangement of the complex is variable, but often appears to consist of a roughly spherical central element enveloped in a cup-shaped outgrowth. The complex is eventually located to one side of the tail flagellum, although extending partially around it.

The spermatid nucleus undergoes a striking series of transformations The chromatin, which initially has a finely granular appearance (Fig. 12.14), becomes more coarsely granular, and appears to consist of particles some 30 nm in diameter (Fig. 12.15). A shallow depression, which becomes the nuclear fossa, forms in the part of the nuclear envelope

adjacent to the centrioles. The granular chromatin becomes attached to the nuclear envelope at this point (Fig. 12.16), and it becomes arranged as a cylinder or pillar extending eventually to the opposite side of the nucleus (Fig. 12.17). On the opposite side, the future anterior end of the sperm, the chromatin attaches closely to the inner membrane of the nuclear envelope, while the outer membrane moves away and lies close to the cell membrane. All the chromatin moves towards the central cylinder, leaving a rim of nucleoplasm, virtually devoid of chromatin, around the lateral surfaces of the nucleus (Figs. 12.18, 12.19). The chromatin progressively becomes more closely packed and dense until the granular substructure can barely be discerned.

Even when nuclear condensation is well advanced, the spermatid contains recognizable Golgi elements, which are often associated with small vesicles. A range of dense vesicles is found around the posterior part of the spermatid nucleus (Fig. 12.20). Many of these are elongate, and often are curved to form horseshoe or ring shapes. While it seems likely that these vesicles are formed by Golgi activity during spermateliosis, no clear indication of their origin was obtained.

Each spermatid comes to contain a single lipid droplet some 250 nm in diameter, closely associated with the mitochondrial complex (Fig. 12.21). Sometimes, especially in early spermatids, the droplet may be surrounded by a rim of electron dense material (Fig. 12.22) in a manner similar to that observed during the early stages of compound yolk fromation in oocytes (see Chapter 6, Section E). Small quantities of

glycogen are also found, usually just posterior to the nucleus (Fig. 12.21).

Aggregations of small dense bodies are also commonly found, often to one side of the nucleus (Fig. 12.23). These bodies are usually 60-80 nm in diameter, are not bounded by a membrane, and consist of a very finely granular material (Fig. 12.24). They are identical to the particulate nuage material found in vitellogenic oocytes (see Chapter 6, Section G).

Many later spermatids are seen to contain a membrane-bound vacuole filled with membranous and other material (Fig. 12.25). This may be the degenerate remains of excess cytoplasm which is lost during spermatellosis. Residual body-like inclusions are also commonly found. No evidence of the sloughing of areas of cytoplasm by spermatids was obtained.

All of these changes take place while the cells are conjoined by cytoplasmic bridges into tetrads. The bridges persist until chromatin condensation is virtually complete. Interestingly, the antero-posterior axis of the spermatid can apparently lie in any orientation with respect to the position of the bridges. Thus bridges can be found at any part of the spermatid surface (Fig. 12.26).

The structure of the mature spermatozoon appears to be identical to that of *Actinia equina*. This sperm was investigated at the ultrastructural level prior to the commencement of the present study, and a description of it has already been published (Larkman and Carter, 1980). For this reason the mature *A. fragacea* sperm will not be described here.

3. Organization of the Testicular Cysts

Slender cytoplasmic processes are often found around the edges of testicular cysts, resting against the basal lamina (Fig. 12.27). Occasionally, such processes are seen to connect with cells whose nuclei are located more centrally within the cyst (Fig. 12.28). It may be that many cells towards the centre of the cysts retain contact with the basal lamina by such slender processes.

The onset of meiosis is always first apparent in cells located near the centre of the cysts, and throughout the development of the cysts there is a gradient, such that more advanced stages are found central to earlier stages. As the cysts near maturity, the outer rim of spermatocytes and spermatogonia gets progressively thinner; eventually it may be reduced to only 2-3 cells thickness (Fig. 12.29). However, over most of the surface of the cyst, this thin layer persists up until spawning time; only in the region of the trophonema (see below) do mature spermatozoa reach the extreme edge of the cyst.

As the number of spermatids and spermatozoa within the cyst increase, they tend to become arranged in strings or columns (Fig. 12.30). Each column is separated from its neighbours by a mass of roughly parallel tail flagella. The columns and the intervening flagella tend to become orientated towards the region of the trophonema, giving the cyst a characteristic fan-like appearance.

Granular amoebocytes often become flattened around the surface of testicular cysts, as they do around developing oocytes (see Chapter 6, Section 1). As in the female gonad, they lie in a precise position, immediately adjacent to the

basal lamina. They often lie in shallow depressions in the surface of the cysts (Fig. 12.31, 12.32).

4. The Male Trophonema

A structure closely corresponding to the female trophonema (see Chapter 7) is found associated with each testicular cyst throughout its development, after entry of the germ cells into the mesogloea (Fig. 12.33). The mode of formation (see Chapter 11), and apparent nutritive function (see Chapter 8) of this structure are very similar to those of the female trophonema, and so it seems reasonable to apply the term trophonema to these structures within the male gonads also.

As in the female, the male trophonema consists of a pore in the mesogloea through which the gonad endodermal epithelial cells extend and contact the germ cells (Fig. 12.34). There is a shallow depression in the surface of the group of germ cells over the area of contact (Figs. 12.35, 12.36). The epithelial arrangement of some of the endodermal cells involved in the trophonema may be obscured, and nuclei may be found in the depression, below the level of the mesogloea. The trophonemal cells may contain accumulations of glycogen and lipid droplets, and bundles of microfilaments are also common (Fig. 12.37).

The interface between the trophonemal cells and the germ cells appears to be less elaborate in the male than in the female. No intercellular junction formation has been observed between the two, and there may be large areas where membrane contact may not be close or regular (Fig. 12.36). The membranes of adjacent trophonemal cells, in contrast, may run closely parallel to each other over large areas (Fig. 12.37).

The mesogloeal lip around the trophonemal pore may be expanded and elaborated, sometimes bearing numerous projections (Fig. 12.38). Microfilament bundles may insert against these projections, and may also insert on the underside of the overhanging mesogloea (Fig. 12.39). Sometimes, well-developed muscle processes can be seen extending through the pore from the gonadal epithelium and inserting against the mesogloea around and beneath the lip region (Fig. 12.40).

Very often, the regions of the trophonemal cells close to the interface with the germ cells contain large dense bodies, which in some cases can be identified as the remnants of germ cells which have presumably been phagocytosed by the trophonemal cells (Figs. 12.36, 12.41). Germ cells at all stages of development may apparently be taken up in this way, although later stages, spermatids and spermatozoa, are found more commonly.

A rim of early stage germ cells persists around most of the periphery of even the most fully developed testicular cysts observed during the present study. However, in the region of the trophonema, this layer may be interrupted. Masses of tail flagella tend to be directed towards the trophonema, and in some cases the trophonemal cells may contact only these flagella (Fig. 12.42). These trophonemal cells may still contain ingested spermatozoa, and numerous residual body-like inclusions, which may represent the remains of previously ingested germ cells.

5. Resorption of Male Gametes

Phagocytosis of germ cells at various stages appears to occur in the region of the trophonema at a relatively low level

throughout much of the life of the testicular cyst (see previous section). Additionally, small numbers of apparently abnormal cells may be found in usually the central regions of cysts, at any time (Fig. 12.43). Granular amoebocytes are sometimes found in the central regions of cysts. These cells often contain numerous residual body-like inclusions (Fig. 12.44), and may perform a scavenging role. They may assist in the removal of degenerate cells.

At around spawning time during June and July, resorption of sperm from the now fully-developed cysts may take place on a very much larger scale. It is difficult to determine, however, whether all the sperm produced by the cyst may be resorbed in this way, or whether only a proportion, possibly those left behind after spawning, are taken up.

During the early stages of resorption, the organization and integrity of the testicular cysts may be lost. They may become less regular in shape, and the arrangement of the sperm into regular strings or columns is obscured (Fig. 12.45). The basal lamina around the outside of the cyst may become thicker, more diffuse and convoluted. Amoebocytes which previously were situated immediately outside the basal lamina may pass through it and contact the germ cells directly (Fig. 12.45). Slender cytoplasmic processes from these amoebocytes may extend between the germ cells, which are largely sperm by this stage, for a considerable distance into the centre of the cyst (Fig. 12.46). Some processes may contain what appear to be the remains of ingested sperm. Amoebocytes already within the cysts also phagocytose sperm cells.

During the periods of major resorption, the uptake of sperm by trophonemal cells continues, apparently at an

increased rate. Some trophonemal cells may contain characteristic clusters of degenerate sperm, consisting of a number of nuclei and mitochondrial remnants surrounded by a coil of tall flagella with an unusually dense appearance (Fig. 12.47).

Similar clusters of degenerate sperm may also be found near the periphery of the cysts, often associated with amoebocyte processes, and in the endodermal epithelial cells around the cysts (Fig. 12.48). They are not restricted to the basal regions of the epithelial cells, but may be found up to and beyond the nuclei (Fig. 12.49). Various stages in what appears to be the breakdown of the sperm clusters can be discerned (Fig. 12.50), and epithelial and trophonemal cells apparently active in the uptake of sperm often contain numerous large residual body-like inclusions (Fig. 12.50). As noted during oocyte resorption (see Chapter 9), the mechanism by which sperm or sperm clusters cross the mesogloea and enter the epithelial cells is not clear.

6. Spermatogenesis in *Cereus pedunculatus*

Only two individuals containing male gonads were found among the *Cereus* samples, and the pattern of spermatogenesis as observed by both light and electron microscopy was similar to that of *A. fragacea*. A number of points of interest did emerge during the course of this study, however, and these will be outlined briefly below. Attention will be concentrated on points of difference between the two species, and on areas which appeared controversial during the study of *A. fragacea*.

The early male germ cells, or prospermatogonia, arise in the endodermal epithelial layer of the gonad, and are similar in both appearance and behaviour to the corresponding cells in

A. fragacea (Fig. 12.53; see Chapter 11). As in *A. fragacea*, even at this early stage these cells possess a flagellum, and it may run either in a membrane-lined groove or channel, or free within the cytoplasm (Fig. 12.54). Sometimes the flagellar membrane can be seen to connect with the membrane of the channel, which may represent a stage in the formation of the channel. Nuage material identical to that found in *Cereus* oocytes is also found in male germ cells (Fig. 12.52). The prospermatogonia migrate from the epithelium into the mesogloea to establish the testicular cysts as in *A. fragacea*. In the early cysts, many cells are found in which the nuclear envelope is poorly defined, incomplete or apparently absent (Fig. 12.54).

The meiotic divisions, although not studied in detail, appear similar to those of *A. fragacea* in many respects. One important difference, however, is that no evidence of incomplete or delayed cytokinesis was obtained. No cells with two nuclei were found, and no cytoplasmic bridges were found linking secondary spermatocytes or spermatids.

The process of spermatelliosis is rather different in the two species. The onset of chromatin condensation in *Cereus* is marked by the appearance of dense threads rather than coarse granules as in *A. fragacea* (Fig. 12.55). The threads appear to be randomly coiled so only short lengths are visible in section. Between the threads, the nuclear material is finely granular. The threads become more extensive and more tightly packed (Figs. 12.56, 12.57), and finally fuse to produce an almost homogeneous dense mass (Fig. 12.58). During condensation, small roughly spherical areas within the nucleus may appear devoid of threads (Figs. 12.56, 12.57), but these are not found once condensation is complete. The nuclear envelope becomes

separated from the condensing chromatin by a sleeve of less dense nucleoplasm around the posterior part of the nucleus, but is closely fitting around the anterior part (Figs. 12.56, 12.57). During chromatin condensation, the nucleus takes on a conical shape, and it may reach 3.5 μm in length, in contrast to the squat nucleus of *A. fragacea* sperm. Horseshoe and ring-shaped vesicles are commonly found around the basal ragions of the nucleus in later spermatids and sperm, and additionally, a row of up to ten rodlets may be found. The rodlets are aligned along the longitudinal axis of the sperm, and are some 70 nm in diameter. They appear to be membrane-bound, and contain finely granular material of moderate density, similar to the contents of the horseshoe vesicles (Figs. 12.59, 12.60).

The spermatid mitochondria fuse to form a single complex, but this is different in appearance to the nearly spherical mass found in *A. fragacea*. In *Cereus*, the complex appears to consist of a pleated sheet (Fig. 12.61), which extends all around the tail flagellum (Fig. 12.62), although it may show a degree of asymmetry. Late spermatids, and cells in which nuclear condensation appears to be complete and so may be considered spermatozoa, may contain considerable quantities of glycogen (Fig. 12.63). This is usually deposited around the mitochondrial complex, although it may extend around the base of the nucleus. The glycogen deposits often appear to give the cell an irregular outline; whether this is a sign of immaturity or whether this is still true of the mature spermatozoon is not known. Occasionally a small lipid droplet is found associated with the mitochondrial complex.

Up to the late spermatid stage, the spermatogenic cells tend to be closely packed together, often having interlocking

shapes and showing close membrane contact. The intercellular spaces found around most male germ cells in *A. fragacea* are not apparent, Once nuclear condensation is complete, however, the cells tend to separate, and become arranged in cords or columns, separated by masses of roughly parallel tail flagella (Fig. 12.64).

The structure of the testicular cysts in *Cereus* appears similar to *A. fragacea*. A structure resembling a trophonema has been observed with the light microscope, but has not been examined in detail by electron microscopy. Resorption of sperm was not observed in *Cereus*, but given the small number of male gonads examined this does not mean that it does not occur.

DISCUSSION

Coelenterate sperm are generally considered to be of the primitive type as proposed by Franzen (1956, 1970). Primitive sperm generally consist of an acrosome, a short head, a mid-piece containing several basically unmodified mitochondria, and a tail which is a normal flagellum. No coelenterate sperm so far described possess a well-defined acrosome (Hinsch, 1974; Schmidt and Zissler, 1979), although some possess so-called pro-acrosomal vesicles anterior to the nucleus. In other respects, most of the hydrozoan and scyphozoan sperm so far described broadly correspond to the primitive form (Miller, 1983). Anthozoan sperm, however, correspond less closely.

Intercellular bridges between spermatogonia are found in many animal species, including mammals (Dym and Fawcett, 1971). They were reported from *Hydra attenuata* (Stagni and Lucchi, 1970), but have not been observed in sea anemones, and were

not encountered during the present study. Incomplete cytokinesis after meiotic divisions seems widespread among coelenterates, however. Tetrads of conjoined spermatids have been reported from many species, including *Hydra fusca* (Schincariol and Habowsky, 1972), *Hydra hymanae* (West, 1978) and the sea anemones *Bunodosoma cavernata* (Dewel and Clark, 1972), *Calliactis parasitica*, *Protanthea simplex*, *Conactinia prolifera*, *Parazoanthus lucifucum* (Lyke and Robson, 1975) and *Aiptasia pallida* (West, 1980). It is surprising that no such bridges were found in *Cereus* gonads during the present study. They were very obvious in *A. fragacea* so it is unlikely that they were simply missed. The spermatids conjoined by bridges in *Hydra fusca* (Schincariol and Habowsky, 1972) show a common orientation of organelles with respect to the position of the bridges. In *A. fragacea*, however, the location of the tail, nucleus and mitochondria of each spermatid appear quite unrelated to the position of the bridges. Zihler (1972) and Tardent (1974) indicate that, in a proportion of cases in *Hydra attenuata*, cytokinesis may not occur at all after one or both meiotic nuclear divisions, giving rise to groups of two or four spermatids developing within a common membrane. Schmidt and Holtken (1980) report a similar occurrence among some Anthozoa. West (1980) also describes this phenomenon in *Aiptasia pallida* but considers it to be an abnormal situation. Intercellular bridges are thought to facilitate the synchronous development of conjoined cells (Dym and Fawcett, 1971).

Synaptinemal complexes are reported to occur in many anthozoan spermatocytes (Schmidt and Zissler, 1979). Those in *A. fragacea* primary spermatocytes appear identical to those found in small oocytes (see Chapters 4 and 5).

The mode of chromatin condensation appears rather different in *A. fragacea* and *Cereus*. In *A. fragacea*, the chromatin first becomes more coarsely granular, and then two plaques of more dense chromatin form, closely applied to the nuclear envelope. One plaque forms at the base of the nucleus adjacent to the centrioles, and the other forms diametrically opposite the first. The plaques appear to act as foci for further condensation, and a longitudinal cylinder of condensing chromatin develops between them. A similar process occurs in the spermatids of *Hydra littoralis* (Weissman *et al*, 1969) and *Hydra hymanae* (West, 1978). The nucleus in *A. fragacea* has a squat cylindrical shape. In *Cereus* spermatids, dense threads form, apparently at random, among the chromatin. As condensation proceeds, the threads become more numerous and more closely packed until a homogeneous dense mass is produced. This form of condensation is similar to that found in *Calliactis parasitica* (Lyke and Robson, 1975) and *Aiptasia pallida*. In all these cases, the resulting nucleus is elongate and conical.

In *Hydra hymanae* (West, 1978), microtubules radiate out from the centrioles and spiral along the length of the spermatid nucleus, in a manner reminiscent of the manchette of higher metazoans (Fawcett *et al*, 1971). West concluded that these microtubules were not involved in sperm head shaping. West (1980) found no microtubules associated with the nucleus in spermatids of the anemone *Aiptasia pallida* and none were observed in *A. fragacea* or *Cereus* spermatids during the present study. Lyke and Robson (1975) also found none in any of the species they studied, and concluded that the final shape of the nucleus must be determined by changes within the nucleus rather than the forces imposed on it from outside.

Fawcett *et al* (1971), likewise concluded that, even in cases where the manchette is well developed, nuclear shaping is likely to be brought about by a controlled pattern of aggregation of DNA and protein during chromatin condensation. Further evidence that this is the case is provided by the observation that, in *A. fragacea*, as chromatin condensation proceeds, the nuclear envelope becomes loosely fitting along the lateral faces. Thus a sleeve of nucleoplasm comes to surround the sides of the chromatin mass. This suggests that the forces changing the shape of the nucleus are generated within it rather than outside. A similar sleeve has been reported from *Hydra hymanae* (West, 1978).

Hydrozoan sperm often contain 4-6 relatively unmodified separate mitochondria (Miller, 1983) and so correspond fairly closely to the primitive condition as put forward by Franzen (1956, 1977). In all sea anemone sperm so far described, with the exception of *Parazoanthus lucifucum*, which is unusual in many respects (Lyke and Robson, 1975), the mitochondria undergo modification and fusion to a greater or lesser extent. In *A. fragacea*, the mitochondria fuse to form a single, large complex, which is located to one side of the tail flagellum. The sperm of *Bunodosoma cavernata* (Dewel and Clark, 1972) and *Tealia felina* (Schmidt and Holtken, 1980) appear similar in this respect. The mitochondrion of *Cereus* sperm forms a ring completely encircling the flagellum and largely filling the cytoplasmic collar. The outer mitochondrial membrane has the form of a smooth, cylindrical ring, while the inner membrane encloses a space shaped like a pleated cord. A similar arrangement has been described for *Calliactis parasitica* (Lyke and Robson, 1975) and *Aiptasia pallida* (West, 1980).

The tail may be placed slightly eccentrically through the complex, but the resulting sperm is much more symmetrical than in *A. fragacea*. Both forms of complex are, however, highly modified forms of mitochondria, and represent a departure from the primitive condition.

A. fragacea spermatids and sperm usually contain a single large lipid droplet, closely associated with the mitochondrial complex, and a very small quantity of glycogen. Lipid droplets were rarely found in *Cereus* spermatids or sperm, but they contain relatively large amounts of glycogen. Presumably both materials are used as a source of energy for sperm locomotion, but the emphasis appears different in the two cases. The presence of a large lipid droplet could also affect the bouyancy of the sperm (Lyke and Robson, 1975).

The nuage material found in spermatogonia and prosperm-atogonia (see Chapter 11) appears identical to that found in early oocytes (see Chapters 4 and 5). The nuage found in later male stages such as spermatids, however, seems identical to the particulate nuage which is common in larger, vitellogenic oocytes (see Chapter 6, Section G). Thus it appears that the form of the nuage alters with advancing stages of germ cell development in a similar way in both sexes. Nuage material was not observed in mature sperm, and has not been reported from the sperm of other coelenterates (Miller, 1983).

West (1978) observed the sloughing of large cytoplasmic droplets containing large inclusions during the final stages of spermatid maturation in *Hydra hymanae*. Such a process was not observed in *Cereus* or *A. fragacea*, and no recognizable cyto-plasmic remnants were found within the testicular cysts. Some *A. fragacea* spermatids contained vacuoles containing

material apparently undergoing breakdown, and residual body-like inclusions were commonly found. It may be that excess cytoplasm undergoes a considerable degree of autolysis and condensation before being expelled from the spermatid. *Hydra* spermatids, like those of other hydrozoans (Roosen-Runge and Szollosi, 1965; Roosen-Runge, 1977) mature among the bases of the ectodermal epithelial cells, and are often in close contact with them. This contact may facilitate the disposal of droplets of residual cytoplasm. In sea anemones, where large numbers of spermatids mature in discrete cysts, with only very limited contact with non-germinal cells, the disposal of excess cytoplasm might pose more of a problem. The early breakdown and compaction of this material may minimise this difficulty.

Amoebocytes come to lie around testicular cysts in much the same way as is found around developing oocytes (Chapter 6, Section I). The possible nutritional function proposed for these cells in the female may also apply in the male gonad. The arrangement of spermatids and sperm in cords or strings in well-developed testicular cysts bears a resemblance to the spermatogenic columns found in some asteroid testes (Walker, 1974, 1980). In asteroids, however, the columns form around a scaffolding of somatic cells. How the cords are formed in sea anemones is not clear, but they do not appear to involve non-germinal cells. Amoebocytes are found within testicular cysts, but they appear to act as wandering phagocytes; they do not enter into close associations with germ cells.

The trophonema found in *A. fragacea* male gonads seems similar in origin and structure to that of the female (see Chapter 7). Wedi and Dunn (1983) reported a plug-like

structure in male gonads of the anemone *Urticina lofotensis*, which they suggested might be homologous with the female trophonema. Otherwise, the existence of a male trophonema has not previously been reported. Schmidt and Holtken (1980) stated that the male mesogloeal gonadal cyst is completely closed in Anthozoa, in contrast to the situation in the female. Such a statement is not consistent with the findings of the present study. In *A. fragacea*, the interface between the trophonemal cells and the germ cells is less elaborate in the male than in the female. Close membrane apposition between the two is less evident, intercellular junctions do not form, and no interdigitation with cytospines can occur. Nevertheless, the autoradiography experiments described in Chapter 8 indicate that male trophonemata are much more active in the uptake and incorporation of small precursor molecules from the external medium than the rest of the gonad epithelium. They may function in the transfer of nutrients to the developing germ cells in the same way as was suggested for the female. The male trophonema also appears to have an additional function of the phagocytosis of germ cells. This may provide another mechanism for the removal of degenerate spermatogenic cells. The trophonema may also participate in the resorption of sperm during and after the spawning period.

The process of resorption of male gametes shows similarities with the resorption of oocytes described in Chapter 9. Some sperm are phagocytosed singly, but most are taken up in spherical clusters reminiscent of the ooplasmic fragments or packets found in the female. As in the female, amoebocytes are clearly involved in the process, but the bulk of digestion of resorbed gamete material takes place in the

gonad epithelial cells. The implications of gamete resorption for the reproductive strategy of the anemone have been discussed in Chapter 9.

Chapter 13

SPAWNING AND EARLY DEVELOPMENT

INTRODUCTION

While fertilization and early development may be regarded as the culmination of the processes of oogenesis and spermatogenesis, they represent areas of the reproductive biology of sea anemones which have received little recent attention. This may be partly due to the difficulty of predicting or inducing the spawning of sea anemones in the laboratory. There have been a number of gross morphological descriptions of embryogenesis in sea anemones and other coelenterates, many dating back to the last century (reviewed by Mergner, 1971). No ultrastructural studies of fertilization or early development have yet been published, and these processes are still poorly understood. Some aspects of spawned egg structure have been investigated, however, including the egg cortical reaction (Dewel and Clark, 1974) and the arrangement of the cytospines (Schroeder, 1982).

It was considered that an overall study of the embryology of *A. fragacea* would represent a major undertaking, beyond the scope of the present study. The following account therefore deals only with those aspects of early development which involve morphological features encountered during the earlier studies of gametogenesis. The findings have been divided into the following sections:

1) Spawning
2) Spawned egg structure
3) Cleavage
4) Breakdown of spawned eggs
5) Blastocoele infilling
6) Uptake of additional sperm
7) Planula structure.

RESULTS

1. Spawning

Batches of 20 large *A. fragacea* individuals were collected from Wembury in May 1982 and from Shaldon in May 1983. The anemones were maintained in the laboratory in two large communal tanks with the sea water changed weekly. The tanks were aerated but no attempt was made to regulate their temperature, and the anemones were kept constantly immersed. Spawning occurred on three occasions, during June and July 1982 and July 1983. On each occasion at least one male and female anemone spawned simultaneously. All three spawnings occurred during spells of hot weather, with water temperatures above 20^{o}C, and within 36 h of changing the sea water. Attempts to repeat these conditions later using the same animals brought no further response.

The tanks were checked at approximately 0900 and 1730 each day. Spawned eggs and sperm were always discovered in the morning, so spawning must have occurred during the evening, night or early morning. The precise time of the onset of spawning could not be determined. On two occasions, a female was still releasing eggs through the mouth when checked. These females were fully open, and their oral discs were covered with eggs and embryos. The eggs were expelled gently in water currents from the mouth, and were not aggregated in mucus or other material.

On one occasion, during June 1982, a female anemone was biopsied for another purpose. The excised gonad released a number of eggs while lying in sea water in a petri dish, and the spawning of the eggs was briefly observed. The gonad was

then fixed and observed by SEM. The surface of this gonad showed a number of differences to the normal, non-spawning gonad described in Chapter 3. In particular, most of the trophonemata appeared as circular grooves (Fig 13.1) instead of the normal simple concavities. Some trophonemata also had a deep, central opening (Fig. 13.2), and, in one case, a broken-off portion of an oocyte was still present in this opening (Fig. 13.3). It appears that the eggs or oocytes are extruded through an opening which forms in the centre of the trophonema. Immediately after release, many eggs are pear-shaped, presumably as a result of constriction during passage through the trophonema, but they soon become spherical.

Gonads were also removed by biopsy from one male and one female anemone which had spawned during the previous night. On EM examination, it appeared that the male gonads still contained many ripe testicular cysts. A small number of cysts, however, appeared disorganized and partially collapsed. It appears that only a small proportion of the sperm had been released on this occasion. The female gonads also contained numerous large oocytes with large, intact nuclei. A small number of trophonemata were found without associated oocytes (Fig. 13.4). It seems possible that these oocytes had been recently spawned. These trophonemal cells appeared normal, except that they rested on the gonad mesogloea rather than the surface of an oocyte. The intercellular junctions joining the apices of the cells appeared intact and normal, so any opening formed during spawning must have re-sealed later. Neither male nor female gonad showed signs of tissue damage or gross disruption.

2. Spawned Egg Structure

Apparently uncleaved eggs were found after each spawning episode. However, no details of the fertilization process were observed, so it was not possible to establish whether or not any individual egg had been fertilized.

a) Surface features of uncleaved eggs

The general surface features of an uncleaved egg are shown by SEM in Fig. 13.5 and TEM in Fig. 13.6. There is no vitelline coat or extracellular investment of any kind around the egg. The egg is covered by tufts of cytospines. These usually lie folded flat against the surface of gonadal oocytes, but after spawning they erect and project out from the surface. The tufts are pyrimadal in shape, with the individual cytospines separated at the base but closely packed towards the tip. Some tufts taper sharply at the base and then continue as a parallel cylinder for some distance (Figs. 13.7, 13.8), while others taper progessively from base to tip (Figs. 13.9, 13.10). The cytospines near the centres of the tufts are usually of greater diameter than those around the periphery. Not all cytospines are involved in tuft formation; some arise individually between the tufts (Fig. 13.9). These tend to be shorter and more slender than those within the tufts. Most tufts show a distinct helical twist, which is obvious under SEM (Figs. 13.8, 13.10), but can also be appreciated using TEM (Fig. 13.6). Not all tufts on a given egg spiral in the same direction. Most spiral anti-clockwise when viewed from the tip, but a small proportion twist in the opposite direction. The rootlets extend straight out from the bases of the cytospines, and so now tend to fan out from the insertions of the tufts (Fig. 13.9), while prior to spawning they tend to run

parallel to each other. Most oocytes show one or several small patches which are devoid of cytospine tufts (Fig. 13.5).

b) Internal structure of uncleaved eggs

The cytoplasm of spawned, uncleaved eggs appears very similar to that of fully grown gonadal oocytes. It appears constant throughout the central portion of the egg, with no obvious regional specialization. The cortical layer still contains large numbers of cortical and fibrillar granules (Figs. 13.7, 13.9), and occasional low-density granules (Fig. 13.11). Vesicles containing large numbers of small, electron-dense particles were also found (Fig. 13.11), which were not observed in gonadal oocytes. Some of these vesicles lack an obvious limiting membrane. Some fibrillar granules (Fig. 13.12) and low-density granules (Fig. 13.13) also contain similar particles, but their significance is unclear.

No male or female pronuclei were observed with the electron microscope.

3) Cleavage

Several rounds of nuclear division appear to take place prior to cytoplasmic cleavage. Nuclei, some apparently in the process of division, were seen around the periphery of eggs which were obviously uncleaved (Fig. 13.14). Where large numbers of nuclei were present, some were also found in more central locations, and the surface of the egg may appear indented (Fig. 13.15). From sections, however, it is difficult to estimate the number of nuclei formed before cytoplasmic cleavage begins. No two, four or eight-cell stages were ever observed. Cleavage furrows appear to form at several points around the egg simultaneously.

The early cleavage furrows are broad (Fig. 13.16), and cytospines and cortical granules are found adjacent to the sides of the furrow. A feltwork of microfilaments is found immediately beneath the plasma membrane at the base of the advancing furrow (Fig. 13.17). Groups of microtubules are also found in various orientations in the cytoplasm close to the base of the furrow. Later, the cleavage furrows become narrower, and the cytospines within them may appear compressed (Fig. 13.18). Intercellular junctions may form at localized areas of contact between neighbouring cells (Fig. 13.19).

Cleavage appears to be total, and divides the embryo into a number of blastomeres each of which contains a single nuclear area (Fig. 13.20). Most nuclei appear to be undergoing mitosis, with condensed chromosomes and no visible nuclear envelope. Interphase nuclei are occasionally found, however (Fig. 13.21). In some cases, the blastomeres show a regular arrangement (Fig. 13.22), but often cleavage appears to be anarchic. A central, fluid-filled space or blastocoele appears early during cleavage (Figs. 13.23, 13.24).

As cleavage proceeds and the blastomeres reduce in size, they become arranged as a columnar epithelium with only narrow intercellular spaces (Fig. 13.25). The nuclei are situated at various levels within the cells, and most are now surrounded by an intact nuclear envelope, suggesting that the rate of mitosis has now slowed. However examples of mitosis are still frequently observed (Fig. 13.26).

Throughout cleavage, the tufts of cytospines remain intact (Fig. 13.22, 13.23), and the composition of the outer layers of cytoplasm remains essentially the same as in the uncleaved egg or the fully grown gonadal oocyte. In particular

cortical granules are still present in apparently undiminished numbers (Fig. 13.25). Low-density granules occur immediately beneath the apical plasma membranes, but in similar numbers to those found in gonadal oocytes. No dramatic change in the cortex of the egg or embryo, indicative of the cortical reaction seen in many other goups, can have taken place at or around the time of fertilization.

During cleavage, the yolk granules and lipid droplets become concentrated in the basal or sub-nuclear portions of the blastomeres (Fig. 13.25). A series of changes in the appearance of these inclusions also takes place, which may represent stages in their utilization.

Initially, a rim of finely granular material forms around the periphery of some of the lipid droplets (Fig. 13.27). At one point on the circumference, this rim becomes thicker, and parallel stacks of membranous material form within it (Fig. 13.28). These stacks become more extensive, and clear areas or spaces form within them, which disrupt their parallel arrangement (Figs. 13.29, 13.30). This process continues until later in development; by the gastrula stage, many lipid droplets have been converted into irregular whorls of trilaminar membrane, some of which still surround unchanged lipid material (Fig. 13.31). Some lipid droplets still remain unaltered, however.

A similar series of changes may affect some of the lipid droplets found within the compound yolk granules. These droplets also come to contain granular material (Fig. 13.32), and clear spaces may form within them. Membranous whorls form around the droplets (Fig. 13.33), and may become extensive (Fig. 13.34). Sometimes more regularly arranged whorls form

around the droplets. Again, only a proportion of compound yolk granules are found undergoing alteration at any one time (Fig. 13.35).

4. Breakdown of Spawned Eggs

After each spawning episode, a small proportion of spawned eggs failed to undergo normal cleavage. In each case, this proportion was estimated at less than 5%. These eggs underwent a process of breakdown and fragmentation in a characteristic manner.

The early stages of this breakdown involve the formation of numerous, roughly spherical blebs around the surface of the egg (Figs. 13.36, 13.37). These blebs are between 2 and 4 um in diameter, and most contain only a sparse, finely granular material and occasional cortical granules (Fig. 13.38). These blebs appear to form as outgrowths from the oolemma, and may become elongate prior to separation (Fig. 13.39). They pass out between the cytospines, and the cytospine tufts become disrupted in the process. Many individual cytospines become swollen or show localized swollen regions (Fig. 13.39). Later, blebs are produced which contain cortical granules and mitochondria, and are thus similar in composition to the cortical ooplasm (Fig. 13.40). Many blebs contain deep invaginations and it seems likely that they undergo further fragmentation soon after formation. These invaginations often seem to originate from bristle-coated pits (Fig. 13.40).

The cortical region of the egg then fragments by the formation of invaginations or furrows in the surface which cut off portions of the ooplasm (Fig. 13.41). Coated pits are again found in the vicinity and at the bases of these furrows,

and may be involved in their initiation (Fig. 13.42). The cytospines become progressively less organized and are eventually shed.

After the loss of the egg cortex, the yolky central portion of the egg also fragments (Fig. 13.43). This process proceeds by the cutting off of peripheral cytoplasmic packets 2-6 μm in diameter. These packets form by the ingrowth of furrows from the surface (Fig. 13.44) and by the formation of clefts within the ooplasm (Fig. 13.45).

The process of fragmentation proceeds until the entire egg has been reduced to a large number of membrane-bound packets. The organelles and inclusions within these packets remain in an unchanged and apparently healthy condition throughout the process. Presumably, however, the packets eventually disperse and degenerate. Overall, this process is strikingly similar to that of centripetal fragmentation undergone by some oocytes while within the gonad, described in Chapter 9.

5. Blastocoele Infilling

Cleavage results in the formation of a roughly spherical hollow blastula. The blastula cells are arranged as a single sheet, with their nuclei about one-third of the cell length from the outer surface. The supranuclear cytoplasm is filled with cortical and fibrillar granules and mitochondria, while the majority of the compound yolk granules and lipid droplets are situated in the basal or sub-nuclear portions of the cells.

In the early blastula, the inner surface of the cell sheet is smooth, and the blastocoele contains only a few membranous sheets and vesicles. The outlines of the individual

cell bases can be clearly traced, and their inner surfaces may bear long microvilli (Fig. 13.46). Later, however, the inner surface becomes more ragged (Fig. 13.47). Membrane-bound packets of yolky cytoplasm detach from the bases of the blastula cells and come to float freely in the blastocoele, although considerable fluid-filled space remains (Figs. 13.49, 13.50). Fig. 13.51 shows a late blastula broken open, showing the single layer of columnar cells and the blastocoele now containing numerous roughly spherical packets of yolky cytoplasm some of which are shown at higher magnification in Fig. 13.51.

Large amounts of membranous material also accumulate in the blastocoele. This may take the form of poorly defined stacks (Fig. 13.53) or whorls (Fig. 13.54). The mode of formation of this material is not clear.

During blastocoele infilling, the embryo becomes slightly elongate and develops a sparse covering of cilia. Such embryos are capable of sporadic swimming movements.

6. Uptake of Additional Sperm

During gastrulation, the embryo takes on a characteristic elongate form shown in Fig. 13.55. It becomes heavily ciliated over its whole surface, and swims actively. Most embryos reach this stage within 24 h of spawning. Gastrulation appears to be brought about by the invagination of cells at the posterior end of the embryo, where a depression forms (Fig. 13.56). The precise mechanism by which this is brought about is not clear, and is beyond the scope of the present study. During this period, however, an interesting and unusual process occurs at the surface of the embryo which is directly relevant to this study and will be described briefly below.

Most gastrulae are found to contain sperm in the apical regions of the outer cell layer. In some areas, considerable numbers of sperm may be found (Fig. 13.57), although the entire surface of the embryo is normally involved to some extent. The sperm are apparently taken up by a process resembling phagocytosis. Slender processes from the surface of the gastrula cells extend out around the sperm heads (Fig. 13.58). The form of initial contact between embryo and sperm is not known, but is likely to involve the cytospines. The processes come to completely surround the sperm head (Fig. 13.59), which is then drawn down into the cell (Fig.13.60) enclosed in a membrane-bound vacuole. In some cases, the sperm tails protrude from the surface of the embryo for some time after the head has been engulfed (Fig. 13.61). Usually, a cytoplasmic extension from the gastrula cell forms a sheath around the flagellum, distinguishing sperm tails from the embryo's own cilia. After ingestion, the microtubules of the tail axoneme very often become coiled within the vacuole (Figs. 13.62, 13.63).

Initially, most sperm nuclei appear normal, and their chromatin is condensed (Fig. 13.64). The sperm mitochondria and other structures, however, usually appear disrupted (Fig. 13.65). Later, the nuclei become coarsely granular and less dense & the nuclear envelope disintegrates (Fig. 13.66). Eventually, the membrane limiting the vacuole around the sperm ruptures, and the sperm remnant is apparently released into the cytoplasm, (Fig. 13.67).

Some sperm found around the surface of gastrula stages appear degenerate prior to ingestion (Fig. 13.68). A common feature of these sperm, both before and after uptake, is the

coiling of the flagellar axoneme microtubules around the sperm head. The individual axonemes are not surrounded by membranes, and the microtubular bundles are located within the sperm cell membrane. Whether these changes occur during the ageing of the sperm, or are the result of interaction with the egg or embryo, is not known.

During gastrulation, the number of fibrillar granules in the apical cytoplasm of the gastrula cells is reduced. Occasionally, fibrillar granules are observed in the process of discharging their contents to the outside of the embryo (Fig. 13.69). This discharge may be responsible for the reduction in their numbers.

7. Planula Structure

With the completion of gastrulation, a hollow, motile planula larva is produced. This stage may be reached within 48 h post spawning. No attempt will be made to describe the overall structure of the planula, but some aspects relevant to this study will be considered.

The body wall of the planula consists of an ectoderm of columnar epithelial cells, and a thinner endoderm of more cuboidal epithelial cells. These layers are separated by a thin layer of mesogloeal material (Figs. 13.70, 13.71). The ectoderm is densely ciliated and bears numerous microvilli some of which may be derived from the egg cytospines. The apices of the ectoderm cells still contain large numbers of cortical granules. Occasional cortical granules are also found in endodermal cells; this may be as a result of morphogenetic cell movements during gastrulation. Fibrillar granules are not present in either cell layer, and no trace of ingested

sperm can be discerned. Compound yolk granules and lipid droplets are common in the endoderm and the basal regions of the ectodermal cells (Fig. 13.72). Many lipid droplets and lipidic inclusions have undergone the series of transformations described above.

Nematocysts and spirocysts appear in both cell layers, but are more common in the ectoderm (Fig. 13.73). They appear to arise in ordinary epithelial cells, which may contain cortical granules. They have not been observed in non-epithelial cells which might correspond to interstitial cells.

The majority of planulae survived with little or no gross morphological change for 12 weeks, when they were discarded. A small proportion settled and metamorphosed into juvenile anemones, starting at five weeks. Some individuals began metamorphosis without settling, but appeared to develop abnormally.

DISCUSSION

In the present study, *A. fragacea* individuals spawned during periods of high water temperature, and possibly in response to an improvement in seawater quality. On each occasion, male and female anemones spawned together. This suggests a mechanism for co-ordinating the spawning of males and females, or at least a response to a common stimulus. Synchronized male and female spawning suggests that fertilization is involved in normal development in *A. fragacea*, and that parthenogenesis or asexual forms of reproduction, which have been proposed for *A. equina* (Gashout and Ormond, 1979; Carter and Thorp, 1979; Orr *et al*, 1982), do not operate in this case.

Synchronized spawning has been reported from other species. Chia and Spaulding (1972) reported that, with *Tealia crassicornis*, at least one individual of each sex spawned on each occasion spawning was observed. Spaulding (1974) suggested that the presence of sperm in the water may stimulate females to release eggs. Dewel and Clark (1972) successfully induced the spawning of male *Bunodosoma cavernata* by adding macerated testis extracts to the aquaria. However, on other occasions, females spawned when kept in isolation, and communally maintained females spawned when males failed to do so (Dewel and Clark, 1974). Female *Stomphia didemon* may also spawn without males (Siebert, 1973).

Gonads removed by biopsy from recently spawned male and female *A. fragacea* still contained large numbers of apparently healthy, fully developed gametes. Thus, not all the gametes are released at once. The remainder could undergo resorption, or repeated spawnings could occur. Spawned *A. fragacea* eggs are negatively bouyant, as are those of *Bunodosoma* (Dewel and Clark, 1974). However, *Tealia crassicornis* eggs float after release (Chia and Spaulding, 1972), as do *Tealia felina* oocytes when teased free from the gonad epithelium (see Chapter 10).

The gonads of *Anthopleura elegantissima* show massive tissue damage after spawning (Jennison, 1979). This was not observed in *A. fragacea*. Here it appears that the eggs are extruded through an opening in the trophonema rather than liberated by breakdown of the gonad epithelium. The well-developed muscle processes and numerous microfilaments found in trophonemal cells (see Chapter 7) may well be involved both in the formation of the opening and the expulsion of the egg through it. The trophonema does not appear to suffer damage

during the process.

The eggs of most marine invertebrates are surrounded by one or more layers of extracellular material, termed variously the vitelline membrane, vitelline layer or vitelline coat. Anderson (1974) argues forcibly that the term vitelline membrane is misleading, and its use should be discontinued. The spawned eggs of *A. fragacea* are unusual in having no extracellular investment of any kind. The absence of any vitelline coat around sea anemone eggs has been reported previously for *Bunodosoma cavernata* (Dewel and Clark, 1974) and *Tealia crassicornis* (Schroeder, 1982). However, Schmidt and Schäfer (1980) report a vitelline membrane around anthozoan oocytes, including those of *Actinia equina*. However, from their published micrographs, it seems possible that they are referring to the basal lamina which surrounds gonadal oocytes and testicular cysts. In *A. fragacea* at least, this layer is not present around spawned eggs. Szmant-Froehlich *et al*(1980) also report a vitelline membrane around eggs of the coral *Astrangia danae*. They describe it as a layer of cortical vesicles just beneath the cell membrane. This layer would therefore appear to be intracellular, and so the use of the term vitelline membrane is perhaps innappropriate. On the other hand, spawned eggs of *Actinostola spetzbergensis* are enclosed in a substantial non-cellular envelope (Riemann-Zurneck, 1976).

The surface architecture of the uncleaved *A. fragacea* egg seems similar to that of *Tealia crassicornis* eggs as described by Schroeder (1982). He also noted the arrangement of the cytospines in pyramidal tufts which he termed spines, and remarked upon their helical twist. Using a fluorescent

labelling technique, he confirmed that the microfilament cores within the cytospines were largely composed of actin. He was not able to resolve individual filaments, however, and so could not detect the regular arrangement of many of the filaments as described in Chapter 6, Section H. Schroeder (1982) remarked upon the very large quantity of actin present near the surface of the egg, which he roughly estimated at some five kilometres of actin microfilament per egg. He suggested that these massive amounts of actin might impart rigidity both to the cytospines themselves and to the egg cortex as a whole. This might serve to maintain the shape of the egg and protect it from mechanical damage. From the present study, it seems likely that the *A. fragacea* cytospines are rigid, and, in the absence of a vitelline coat, may well serve to protect the egg surface from abrasion and damage. However, it is not clear that the egg cortex shows any great degree of rigidity; the egg undergoes considerable distortion during release from the gonad, and the curvature of the surface changes during cleavage without changing its composition. Chia and Spaulding (1972) also report that *Tealia crassicornis* eggs seem soft and flatten "into a pancake shape" when placed on a slide.

Schroeder (1982) noted a small area on each *Tealia* egg which was devoid of cytospine tufts or spines. He suggested that this might represent the site of polar body formation. Similar areas occur on *A. fragacea* eggs, but their significance is unclear. Siebert (1973) noted that sperm may become trapped among the cytospines of *Stomphia didemon* eggs, and this was also observed during the present study. Unless the sperm are specifically attracted to the small areas of the egg surface

which are devoid of cytospines, it seems likely that the initial contact between sperm and egg may occur on the cytospines rather than on the intervening oolemma. The cytospines may thus be the site of specific receptor molecules, which are thought to be involved in sperm/egg recognition and binding in other invertebrate groups (Summers and Hylander, 1975; Glabe and Vacquier, 1978).

Although polar body formation was not observed in *A. fragacea* it seems likely that meiosis is completed soon after release from the gonad. All the gonadal oocytes observed during the present study had intact germinal vesicles, but none were observed in spawned eggs. A similar situation has been reported for most other anemone species studied (Gemmill, 1921; Nyholm, 1943, 1949; Chia and Spaulding, 1972; Spaulding, 1974; Dewel and Clark, 1974; Jennison, 1979; Szmant-Froehlich *et al*, 1980).

Cleavage in *A. fragacea* appears to follow a very similar pattern to that described for *Bolocera tuediae* by Gemmill (1921). He estimated that the zygote nucleus gave rise to at least 24 daughter nuclei before the onset of cytoplasmic cleavage. Appellöf (1900) estimated that 16 such nuclei were produced in *Urticina*. It was not possible to obtain a reliable estimate for *A. fragacea*, although there would appear to be at least 16. Spaulding (1974) suggested that the larger the anemone egg, the greater the number of nuclei produced before segmentation begins. In some large anemone eggs, however, cleavage may follow a different pattern. Chia and Spaulding (1972) found that, with the relatively large eggs of *Tealia crassicornis*, early cleavage was meroblastic and confined to the animal pole. A similar situation has also been reported for *Stomphia*

(Siebert, 1973). In *Epiactis prolifera*, a large number of nuclei are formed before the onset of cleavage. These nuclei become arranged in two distinct layers around the periphery of the egg, and when cytoplasmic segmentation occurs, an embryo with two cell layers is formed directly (Dunn, 1975).

Cleavage in many animal eggs, and cytokinesis in many other cell types, is brought about by a 'contractile ring' of numerous microfilaments extending around the circumference of the cell (Bluemink, 1970; Beams and Kessel, 1976). The microfilaments are largely composed of actin (Schroeder, 1969, 1973), and myosin is also present (Fujiwara and Pollard, 1976). Antibodies against myosin can inhibit cleavage (Mabuchi and Okuno, 1977), so it seems likely that the contraction is generated by the interaction of actin and myosin filaments. Several such contractile rings appear to form simultaneously at different points around the surface of the *A. fragacea* egg, but their appearance is otherwise similar to those of other species. In *A. fragacea*, bundles of microtubules were also found in the cytoplasm adjacent to the advancing cleavage furrows. Their significance is unclear, and, in eggs of other species, microtubules are not thought to contribute directly to the ingression of the cleavage furrows.

The process of breakdown of spawned eggs is very similar to the breakdown of large oocytes within the gonad, as described in Chapter 9. After spawning, however, breakdown must be regarded as pathological, since the products of breakdown are not resorbed, and so can not be of benefit to the adult. It may be the result of a failure to complete meiosis successfully or a failure of the fertilization process, either by non-fertilization or polyspermic fertilization. Alternatively,

the eggs may be defective in some other way, or suffer damage during or after spawning. In any case, breakdown appears to follow a controlled pattern of fragmentation, with little structural alteration of the individual ooplasmic inclusions. The proportion of eggs breaking down in this way was always very low.

The process of blastocoele infilling also involves the controlled fragmentation of cells. It results in the production of membrane-bound packets of yolky cytoplasm which are essentially identical to those formed during oocyte and egg breakdown. Thus it appears that a similar mechanism operates during normal development and during a degenerative process. Blastocoele infilling by anucleate, yolky fragments has been reported during the development of several anthozoan species (Mergner, 1971). Mergner suggests that the shedding of yolky material may render the blastula cells more freely motile, and so facilitate the morphogenetic cell movements required during gastrulation.

At fertilization, the eggs of most animals are faced with the need to permit the entry of a single sperm, but then rapidly undergo some alteration which prevents the entry of further sperm. Polyspermy, the entry of more than one sperm into the egg, usually results in the death or abnormality of the resulting embryo. Mechanisms of polyspermy prevention have been investigated intensively in recent years (see reviews by Schuel, 1978; Shapiro and Eddy, 1980; Dale and Monroy, 1981; Guraya, 1982). The eggs which have been studied most thoroughly in this respect are those of some echinoderms, and in particular those of sea urchins. The sea urchin egg appears to possess two mechnisms to prevent the entry of additional or

supernumary sperm. Very soon after the entry of the first sperm, there is an electrical depolarization of the egg plasma membrane, which makes it more difficult for subsequent sperm to bind and fuse with it. This electrical block is rapid, but is not absolute, and may be of only limited duration (Nuccitelli and Grey, 1984). A more complete and permanent block is provided by the so-called cortical reaction. The cortical reaction involves the discharge of the contents of the cortical granules to the outside of the egg. The contents are released into the space between the oolemma and the vitelline coat, and bring about several changes, including the following:-

a) The separation or elevation of the vitelline coat away from the oolemma, and

b) the chemical alteration of the vitelline coat, rendering it less attractive and impermeable to sperm (Schuel, 1978).

The elevated, altered vitelline coat is often called the fertilization envelope, and provides a total and permanent barrier to the entry of further sperm into the egg. Its formation is relatively slow, however, taking over 60 s in *Arbacia punctulata* (Schuel, 1978), and it is now widely accepted that both blocks are necessary if polyspermy is to be effectively prevented. The rapid, electrical block has been investigated in detail in only relatively few species, but the cortical reaction has been described from a number of groups, including several invertebrate phyla, teleosts, amphibians and mammals (Guraya, 1982).

A dramatic cortical reaction has been described in some detail for the sea anemone *Bunodosoma cavernata* (Dewel and Clark, 1974) and reported more briefly from *Metridium senile* (Clark and Dewel, 1974). In both of these species, there is a massive discharge of cortical granules. In *Bunodosoma*, the discharge involves the coalescence of neighbouring granules, their fusion with the oolemma and the release of their contents to the outside of the egg. Portions of the cortical ooplasm, containing mitochondria, lipid droplets and endoplasmic reticulum may also be shed during this process, and the egg apparently undergoes a significant reduction in diameter. The arrangement of the cytospines is also disrupted, and their continuity with the oolemma is "at the very least obscured" and may be lost altogether. However, it was reported that only those eggs which displayed such a reaction went on to cleave and develop normally.

During the present study, the events associated with the entry of the first sperm into the egg in *A. fragacea* were never observed. However, the examination of multinucleate eggs and cleavage stage embryos shows that the surface features of the egg remain essentially unchanged. Cortical granules are present in apparently undiminished numbers through to at least the planula stage, and at no time do the cytospines appear disrupted. No dramatic cortical reaction can have taken place after fertilization.

Schroeder (1982) did not follow early development in *Zealia crassicornis* in detail, but he did note that no differences in surface organization were apparent between fertilized and unfertilized eggs, and that the cytospines were retained intact during early development. Chia and Spaulding

(1972) also found that the cytospines in *Tealia* persisted throughout cleavage. It thus appears that no cortical reaction comparable to that described for *Bunodosoma* occurs in this anemone.

A number of other examples have been reported of invertebrate eggs in which either no visible cortical reaction occurs or where substantial numbers of cortical granules persist into later developmental stages. These examples include *Hydra* (Honegger, 1983), *Mytilus* (Humphreys, 1967) *Spisula* (Longo, 1976), *Haliotis* (Lewis *et al* 1982) *Chaetopterus* (Anderson, 1974) and *Urechis* (Paul, 1975; Gould-Somero and Holland, 1975). The role of the cortical granules in these cases remains to be determined. Humphreys (1967) suggested that the prolonged, slow discharge of cortical granules observed in *Mytilus* might serve to maintain the vitelline coat during embryonic development. In the absence of any form of vitelline coat, it is difficult to envisage the function of cortical granule discharge in sea anemones. It seems possible that the granule contents would be rapidly dispersed in the surrounding sea water.

Some mechanism for polyspermy prevention must operate in *A. fragacea*. The eggs spawned in closed tanks in the laboratory were exposed to apparently very high concentrations of sperm, yet the vast majority developed normally, and no supernumary sperm were observed in cleavage stage embryos. The nature of this mechanism is at present unknown, but it may be similar to the fast electrical block found in sea urchins and described above. The fact that gastrula stage embryos can take up large numbers of sperm might indicate that this block, which has operated effectively throughout cleavage, may later fail and allow sperm to enter the embryo. By the gastrula stage,

however, the entry of additional sperm may pose little threat to the genetic constitution of the embryo, and in *A. fragacea* the ingested sperm appear to be rapidly broken down.

It is not certain that the uptake of sperm by gastrulae is a selective and specific process; it is possible that other particles of similar size might be ingested in the same way. No particles other than sperm were observed being taken up by gastrulae, but, in the closed tanks in which the embryos were maintained, sperm might be the most abundant particles of suitable size available. However, if the uptake is not specific, it is unclear why a relatively early embryo which has barely begun to mobilize its yolk reserves should be active in the uptake of particles from the external environment. Sperm were not apparently taken up by post-gastrula stages, but this could merely reflect the reduced availability of sperm to these later stages. The ageing sperm tend to settle to the floors of the tanks, while the now motile larvae swim actively and spend less time on the bottom.

The roles of the fibrillar and cortical granules during early development remain unclear. The fibrillar granules decrease in number during embryonic life and are absent from planulae. Some of these granules appear to be discharged to the exterior, although their function is unknown. Most cortical granules persist apparently unchanged until at least the planula stage, so it is difficult to envisage any major function for them during embryogenesis.

While the development of nematocysts in the planula was not studied in detail, it would appear that they can arise in unmodified ectodermal epithelial cells. In *Hydra* and other

hydroids, nematocysts develop in non-epithelial cells derived from interstitial cells (Bode and David, 1978). The apparently different mode of nematocyst formation found in larval sea anemones highlights the need for a better understanding of the role, if any, of interstitial cells in anthozoan species.

Chapter 14

GENERAL DISCUSSION

Detailed discussion of the results of the present study and of the relevant literature has been covered in the discussion sections of individual chapters. Only some more general considerations and some suggestions as to the direction future work on this topic might follow will be presented here.

In most areas of coelenterate biology, it is difficult to avoid some speculation on the question of how 'primitive' or 'advanced' a particular process or structure may be. In the case of sea anemone gametogenesis, the overall picture emerging from the earlier literature, principally from studies with the light microscope, as reviewed by Campbell (1974a, 1974b), is consistent with what might be expected from a primitive metazoan. The gametes were thought to arise as required from undifferentiated, pluripotent interstitial cells, and develop with little or no involvement of somatic accessory cells. The absence of accessory cells indicated that vitellogenesis was wholly autosynthetic, i.e carried out entirely within the oocyte itself. The release of gametes was thought to be brought about by the simple break-down of the fabric of the gonad. When gametogenesis in *A. fragacea* is examined in detail with the electron microscope however, a rather different pattern perhaps emerges.

The origin of the germ cells in *A. fragacea* is still unclear, but it is by no means certain that they derive from pluripotent interstitial cells. There is some evidence, from the occurrence of nuage material, that some degree of separation of the germ cell line from the somatic cells may occur. Evidence for a similar separation is beginning also to emerge for *Hydra* (Littlefield, 1984). It is not clear

how the germ cells become distributed through the numerous gonads during development, but in *A. fragacea* the gonads appear to be retained from year to year. A degree of separation of germ line cells would make germ cell differentiation less of an 'epigenetic' process, and would bring it at least some way closer to the situation found in higher invertebrates and vertebrates (Nieuwkoop and Satasurya, 1979, 1981).

The present study has indicated that after entry into the mesogloea, the gametes do not develop wholly independently but are closely associated with both granular amoebocytes and the cells of the trophonema. The trophonema in particular appears to be highly specialized for an accessory role, especially during oogenesis. The trophonemal cells are intimately associated with the surface of each developing oocyte, and appear to be involved in nutrient transfer. Trophonemal cells are also more active in protein synthesis than the remainder of the gonad. This greater synthetic acitivity may be the result of a general increase in the production of a wide spectrum of proteins, but it is possible that trophonemal cells produce a protein or proteins specifically for export to the growing oocyte. Such an activity would constitute the heterosynthetic or extra-oocytic production of yolk protein, often considered to be an 'advanced' feature during oogenesis (Eckelbarger, 1983). The oocytes contain a highly developed system of rough endoplasmic reticulum, and undoubtedly are engaged in autosynthetic or intra-oocytic yolk production, but it seems likely that hetero-synthesis also plays a part. Such a form of 'mixed' yolk synthesis may be more widespread than has previously been

appreciated.

The eggs of most marine invertebrates are surrounded by an extracellular investment such as a vitelline coat, and sometimes by additional jelly layers (e.g Anderson, 1974). These investments serve to protect the egg from damage after spawning and during early development, and are often also involved in polyspermy prevention (e.g Schuel, 1978). However, the presence of such a layer may also have disadvantages. If it is present during oogenesis, it may hinder the passage of nutrients into the oocyte, and after spawning it may hinder the entry of the fertilizing sperm. To overcome these difficulties, processes from the oocyte surface and surrounding accessory cells may extend through these layers to permit nutrient transfer during oogenesis, and most invertebrate sperm possess an acrosome, which locally softens the extracellular coat and permits sperm penetration. The eggs of *A. fragacea* do not have any form of extracellular investment, and so must achieve the necessary degree of protection from damage in some other way. The surface of the egg is covered by apparently rigid tufts of cytospines which may perform this function, and may actually assist rather than hinder the process of nutrient uptake during oogenesis. The cytospines are elaborate and highly ordered structures, which are larger and more complex than the surface features found on the eggs of most higher animals. The absence of an extracellular investment around the egg is presumably correlated with the absence of acrosome in the sperm (Larkman and Carter, 1980). The mechanisms of polyspermy prevention in *A.fragacea* are not known, but are apparently very different from those found in most other groups. That the alternative mechanisms

employed by *A. fragacea* are effective is shown by the very high success rates in producing apparently normal embryos and larvae after spawnings in the laboratory. The vast majority of eggs developed normally in spite of culture conditions which left much to be desired by conventional standards for rearing marine organisms.

In *A. fragacea*, the release of eggs from the gonad during spawning appears to be a precisely controlled process in which the somatic trophonemal cells are closely involved (see Chapter 13). The gonad epithelium does not appear to suffer damage during the process. This would appear to be a more sophisticated mechanism than that reported for some other anemones, where the gonad epithelium breaks down to release the gametes (Jennison, 1979).

In *A. fragacea*, germ cells may break down at almost any stage during their development, and, at least on some occasions, fully grown oocytes may break down in large numbers. When viewed by light microscopy, the breakdown of large oocytes appears to be a haphazard, degenerative process. When examined in detail by electron microscopy, however, it is seen to be a regulated process, involving the co-operative activity of the gonad epithelial cells, granular amoebocytes and the oocytes themselves. This form of breakdown results in the orderly re-cycling of large quantities of oocyte material without damage to the gonad epithelia. Sperm may be resorbed by a similar mechanism in the male gonad.

Thus the overall pattern of gametogenesis in *A.fragacea* which has emerged rom the present study suggests that it is a more complex and better regulated process than perhaps was appreciated previously. In particular, it involves considerable

co-operative interaction between germ and somatic cells. In this respect, it perhaps goes some way towards the 'advanced' forms of gametogenesis found in higher animals. On the other hand, the appearance of the compound yolk granules is very similar to structures found in the eggs and gemmules of sponges.

A number of points arising from the present study appear to warrant further investigation employing a wider range of techniques than used here. The occurrence and role of interstitial cells in sea anemones is in urgent need of clarification. If the patterns of cell proliferation throughout the anemone could be established, perhaps using tritiated thymidine autoradiography at the electron microscope level, as outlined in Chapter 4, this might improve our understanding of an important problem in modern coelenterate biology.

The extent of the role played by the trophonemal cells and amoebocytes in the transfer of nutrients to the developing germ cells needs further study. Some nutrient molecules may be channelled through the trophonema, some through amoebocytes and some may be taken up directly from the mesogloea by the oocyte surface. In some polychaete annelids, there are a number of pathways by which the various nutrients reach the oocyte, only some of which involve accessory cells (Dhainaut *et al*, 1984). The autoradiographic studies described in Chapter 8 could be extended to include a wider range of labelled molecules, and some should be performed at the electron microscope level. Another possibility which might be explored is that the trophonemal cells may synthesize messenger RNA for export to the oocyte. This RNA might then be translated using the oocyte's own, well-developed synthetic machinery, or stored for use during early development.

The supply of mRNA to oocytes by nurse cells is known to occur in some insects (Paglia *et al*, 1976). Patterns of RNA synthesis could be followed using autoradiography after exposure to radiolabelled uridine.

The gonads and oocytes of *A. fragacea* might also serve as models for the study of several cellular structures and processes which are important in modern cell biological research. The oocyte cytospines would appear to be highly suitable structures for the study of the organization of the cytoskeleton in microvilli and allied structures. Cytospines are larger and more precisely arranged than the microvilli of vertebrate brush border epithelia, which are the standard subjects for research of this kind. The oocyte surface also provides a system free from the cell boundaries and inter-cellular junctions found in vertebrate epithelia, and might make an interesting comparison. Deep etching techniques might reveal more of the cytoskeletal organization associated with the cytospines than could be distinguished by conventional methods during the present study.

The trophonema and underlying regions of the oocyte might make a suitable system for the study of endocytosis and associated problems of membrane turnover. Two membrane surfaces are involved in endocytosis in this system, the apical surface of the trophonemal cells, and the oocyte surface adjacent to the trophonema. The trophonemal cells are involved in the uptake of presumably a wide range of different protein molecules from the gastrovascular fluid (see Chapter 8). The results of horseradish peroxidase uptake experiments suggest that the proteins taken up in this way are processed & degraded by the trophonemal cells.

The apical regions of these and other gonad epithellal cells possess numerous lysosomes and an elaborate transfer tubule system, possibly for this purpose. The ooplasm adjacent to the trophonema on the other hand, contains only large numbers of vesicles. Lysosomes and a transfer tubular system are not in evidence in this location. This may be because the oocyte is taking up only a more limited range of protein molecules, which might require relatively little further processing before utilization within the oocyte. The scale of endocytosis in this region of the oocyte, as evidenced by the numbers of vesicles found there, would appear to be much greater than occurs in the cultured mammalian cells or hepatocytes often used in studies of endocytosis. Thus the associated problems of membrane turn-over and receptor re-cycling may also be greater. Modern immuno-labelling techniques might be used to good effect for the study of these mechanisms in this situation.

During the present study, cytological aspects of oocyte degeneration and fragmentation were studied in some detail (see Chapter 9), and it became apparent that resorption of gametes could occur on a large scale. The very small samples examined in this sort of ultrastructural study, however, do not permit conclusions to be drawn about the situation in the population as a whole. A study of the phenomenon from a more ecological viewpoint is required to assess the impact and importance of gamete resorption as a reproductive tactic. More definite knowledge of the longevity of *A. fragacea* individuals in the field could be obtained by estimating adult mortality rates, and the extent of resorption in the population could be monitored by larger scale sampling, or possibly by biopsy of anemones *in situ*. More data on these two parameters

might confirm the rather speculative argument on the role of gamete resorption in a long-lived animal presented in the Discussion section of Chapter 9.

The fact that the moment of fertilization in *A.fragacea* was never observed was a major disappointment during this study. However, armed with a knowledge of the gametogenic cycle and of the conditions which appear to promote spawning, there seems no reason why future attempts to induce and observe spawning in the laboratory should not meet with success. Processes such as polar body formation and sperm/egg interaction could then be observed. Given the current high level of research interest in fertilization mechanisms in animals generally, a determined effort to achieve this might well be worthwhile.

Much recent research effort in invertebrate reproduction has been directed towards understanding the mechanisms by which gametogenesis and spawning are controlled. The roles of neurosecretory and endocrine control systems have been investigated in several invertebrate phyla, including annelids, molluscs, insects and echinoderms (e.g De Loof, 1984; Voogt *et al*, 1984; Schroeder, 1984; Raabe, 1984). Gametogenesis in *A. fragacea* appears to be a well-regulated process, co-ordinated across a number of discrete gonads. The mechanisms by which control is achieved, however, are entirely unknown. Sterols are known to occur in sea anemones (Voogt*et al*, 1974; Sica *et al*, 1981), but their function is not known. Nervous elements were not observed within the gonads at any stage during the present study. One possible contribution to the future study of control mechanisms is the finding that anemone gonads can be maintained in culture. With some

additions to the culture medium, it seems possible that the culture method used in the experiments described in Chapter 8 could be used to maintain gonads for a matter of weeks. The culture of gonads in a situation where they are not subjected to extrinsic controlling influences may shed light on the role of such influences, and makes possible the experimental testing of the effect of chemicals and tissue extracts on gamete development. Anemone gonads appear to be relatively easy to maintain in culture. The fact that they are normally exposed to the external environment in the form of the gastrovascular fluid, and can take up a range of nutrients from it, may contribute to this. Anemones appear to be long-lived animals, and so must be highly resistant to infection by microorganisms. In the absence of a definite circulatory system, it is likely that much of their defence against infection operates at a local level. Thus isolated organs such as gonads may retain some ability to resist infection, which again may contribute to their prolonged survival in culture.

The only other large scale ultrastructural studies of anthozoan gametogenesis published are those of Schmidt and co-workers (Schmidt and Zissler, 1979; Schmidt and Schafer, 1980; Schafer and Schmidt, 1980; Schmidt and Holtken, 1980; Schafer, 1983). Their approach has been to examine gonads from a very large number of different species, with a view to using differences in gamete ultrastructure as indicators of phylogenetic and taxonomic relationships. Their descriptions of the processes of oogenesis and spermatogenesis have been pieced together using fragments of information from different species. Such an approach is not without its disadvantages, however, and several of the findings of the present study are

inconsistent with theirs. Comparative studies of more than one species are, of course, valuable, but when dealing with a complex process such as gametogenesis, individual species must be studied in some depth before electron microscope results can be interpreted reliably. Unless complications such as gamete degeneration and resorption are identified and their consequences appreciated, even good-quality electron micrographs are likely to be subject to misinterpretation.

The present study represents an attempt to subject the entire process of gametogenesis in a single species of sea anemone to detailed ultrastructural examination. Hopefully it makes some contribution towards the better understanding of an important process in an interesting group of animals.

REFERENCES

Adiyodi, K.G. and Adiyodi, R.G. (1983a)
"Reproductive Biology of Invertebrates, Volume 1. Oogenesis, Oviposition and Oosorption". London, John Wiley.

Adiyodi, K.G. and Adiyodi, R.G. (1983b)
"Reproductive Biology of Invertebrates, Volume II. Spermatogenesis and Sperm Function". London, John Wiley.

Aizenshtadt, T.B. (1974)
Investigation of oogenesis in *Hydra*. 1. Ultrastructure of interstitial cells at early stages of their transformation into oocytes.
Sov. J. Dev. Biol., 5: 9-18.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983)
"Molecular Biology of the Cell". New York, Garland Publishing Inc., 1146 pp.

Al-Mukhtar, K. and Webb, A.C. (1971)
An ultrastructural study of the primordial germ cells, oogonia and oocytes in *Xenopus laevis*.
J. Embryol. exp. Morphol., 26: 195-217.

Anderson, E. (1968)
Oocyte differentiation in the sea urchin *Arbacia punctata*, with particular reference to the cortical granules and their participation in the cortical reaction. J. Cell. Biol., 37: 514-539.

Anderson, E. (1969)
Oocyte-follicle cell differentiation in two species of amphineurans (Mollusca), *Mopalia mucosa* and *Chaetopleura apiculata*. J. Morphol., 129:89-125.

Anderson, E. (1974)
Comparative aspects of the ultrastructure of the female gamete.
Int. Rev. Cytol., Suppl. 4; 1-70.

Anderson, E. and Huebner, E. (1968)
Development of the oocyte and its accessory cells of the polychaete, *Dioptera cuprea* (Bosc).
J. Morphol., 126: 163-197.

Anderson, L.M. and Telfer, W.H. (1970)
Extracellular concentrating of proteins in the *Cecropia* moth follicle.
J. Cell Physiol., 76: 37-53.

André, J. and Rouiller, C. (1957)
L'ultrastructure de la membrane nucléaire des ovocytes de l'araignée (*Tegenaria domestica* Clark)
In F.S. Sjostrand and J. Rhodin (Eds.) "Proceedings of the European Conference on Electron Microscopy, Stockholm, 1956. New York, Academic Press, pp. 162-164.

Appellöf, A. (1900)
Studien über Aktinien-Entwicklung. Bergens Museum Aarbog, 1900, pp. 1-99.

Ashworth, J.H. and Annandale, N. (1904)
Observations on some aged specimens of *Sagartia troglodytes*, and on the duration of life in Coelenterates.
Proc. Roy. Soc. Edinburgh, 25: 295-308.

Baccetti, B. and Afzelius, B.A. (1976)
"The Biology of the Sperm Cell"
Basel: Karger, 254 pp.

Balinsky, B.I. (1966)
Changes in the ultrastructure of amphibian eggs following fertilization.
Acta. Embryol. Morphol. Exp., 9: 132-154.

Barer, R., Joseph, S. and Meek, G.A. (1960)
The origin and fate of the nuclear membrane in meiosis. Proc. Roy. Soc. Lond., B152: 353-366.

Barnes, B.G. and Davis, J.M. (1959)
The structure of the nuclear pores in mammalian tissue. J. Ultrastruct. Res., 3: 131-146.

Bauer, P.S. and Stacey, T.R. (1977)
The use of PIPES buffer in the fixation of mammalian and marine tissues for electron microscopy.
J. Microsc., 109: 315-327.

Beams, H.W. and Kessel, R.G. (1963)
Electron microscope studies on developing crayfish oocytes with special reference to the origin of yolk.
J Cell Biol., 18: 621-649.

Beams, H.W. and Kessel, R.G. (1974)
The problem of germ cell determinants.
Int. Rev. Cytol., 39: 413-479.

Beams, H.W. and Kessel, R.G. (1976)
Cytokinesis: a comparative study of cytoplasmic division in animal cells. Am. Sci., 64: 279-290.

Beams, H.W. and Kessel, R.G. (1983)
Cnidaria. In K.G. Adiyodi and R.G. Adiyodi (Eds.) "Reproductive Biology of Invertebrates, Vol. 1. Oogenesis, Oviposition and Oosorption". London, John Wiley. Chapter 2.

Benayahu, Y. and Loya, Y. (1983)
Surface brooding in the Red Sea soft coral *Parerythropodium fulvum fulvum* (Forskål, 1775). Biol. Bull., 165: 353-369.

Benayahu, Y. and Loya, Y. (1984)
Life history studies on the Red Sea soft coral *Xenia macrospiculata* Gohar, 1940. 1. Annual dynamics of gonadal development. Biol. Bull., 166: 32-43.

Bilinski, S. (1977)
Oogenesis in *Acerentomon gallicum* Jonescu (Protura). Previtellogenic and vitellogenic stages. Cell Tiss. Res., 179: 401-412.

Billet, F.S. (1979)
Oocyte mitochondria. In D.R. Newth and M. Balls (Eds.) "Maternal Effects in Development". Cambridge University Press, pp. 147-166.

Billet, F.S. and Adam, E. (1976)
The structure of the mitochondrial cloud of *Xenopus laevis* oocytes. J. Embryol. exp. Morphol., 33: 697-710.

Blinzinger, K., Rewcastle, N.B. and Hager, H. (1965)
Observations on prismatic-type mitochondria within astrocytes of the Syrian hamster brain. J. Cell Biol., 25: 293-303.

Blobel, G. and Dobberstein, B. (1975)
Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components.
J. Cell Biol., 67: 852-862.

Blobel G. and Sabatini, D. (1971)
Dissociation of mammalian polyribosomes into subunits by puromycin.
Proc. Natl. Acad. Sci. USA., 68: 390-394.

Bluemink, J.G. (1970)
The first cleavage of the amphibian egg. An electron microscope study of the onset of cytokinesis in the egg of *Ambystoma mexicanum*.
J. Ultrastruct. Res., 32: 142-166.

Bode, H.R. and David, C.N. (1978)
Regulation of a multipotent stem cell, the interstitial cell of *Hydra*.
Prog. Biophys. Molec. Biol., 33: 189-206.

Boelsterli, U. (1975)
Notes on oogenesis in *Tubularia crocea* Agassiz (Athecata, Hydrozoa).
Pubbl. Staz. Zool. Napoli, 39 (Suppl.), 53-66.

Boelsterli, U. (1977)
An electron microscopic study of early developmental stages, myogenesis, oogenesis and cnidogenesis in the anthomedusa, *Podocoryne carnea* M. Sars.
J. Morphol., 154: 259-290.

Bondi, C. and Facchini, L. (1972)
Observations on the oocyte ultrastructure and vitellogenesis of *Branchiobdella pentodonta* Whitman.
Acta Embryol. Exper., 4: 225-241.

Booth, A.G. and Vanderpuye, O.A. (1983)
Structure of human placental microvilli. In R. Porter and G.M. Collins (Eds.) "Brush Border Membranes". London, Pitman Books Ltd., pp. 180-194.

Bouillon, J. (1968)
Introduction to coelenterates. In M. Florkin and B.T. Scheer (Eds.) "Chemical Zoology", New York, Academic Press, pp. 81-147.

Boury-Esnault, N. and Doumenc, D.A. (1979)
Glycogen storage and transfer in primitive invertebrates: Demospongea and Actinaria. In C. Levi and N. Boury-Esnault (Eds.) "Biologie des Spongiaires". Paris: CNRS., pp. 181-192.

Brien, P. (1953)
La pérennité somatique. Biol. Rev., 28: 308-349.

Brien, P. (1954)
Origine et localisation des cellules germinales chez les Hydroides gymnoblastiques.
Mem. 3E Cong. Nat. Sci., 6: 3 pp.

Brien, P. and Reniers-Decoen, M. (1955)
La signification des cellules interstitielles des hydras d'eau douce et la problème de la réserve embryonnaire.
Bull. Zool. France Belg., 89; 258-325.

Browder, L.E. (1980)
"Developmental Biology". Philadelphia, Saunders, 602 pp.

Booth, A.G. and Vanderpuye, O.A. (1983)
Structure of human placental microvilli. In R. Porter and G.M. Collins (Eds.) "Brush Border Membranes". London, Pitman Books Ltd., pp. 180-194.

Bouillon, J. (1968)
Introduction to coelenterates. In M. Florkin and B.T. Scheer (Eds.) "Chemical Zoology", New York, Academic Press, pp. 81-147.

Boury-Esnault, N. and Doumenc, D.A. (1979)
Glycogen storage and transfer in primitive invertebrates: Demospongea and Actinaria. In C. Levi and N. Boury-Esnault (Eds.) "Biologie des Spongiaires". Paris: CNRS., pp. 181-192.

Brien, P. (1953)
La pérennité somatique. Biol. Rev., 28: 308-349.

Brien, P. (1954)
Origine et localisation des cellules germinales chez les Hydroides gymnoblastiques.
Mem. 3E Cong. Nat. Sci., 6: 3 pp.

Brien, P. and Reniers-Decoen, M. (1955)
La signification des cellules interstitielles des hydras d'eau douce et la problème de la réserve embryonnaire.
Bull. Zool. France Belg., 89; 258-325.

Browder, L.E. (1980)
"Developmental Biology". Philadelphia, Saunders, 602 pp.

Buisson, B. and Franc, S. (1969)
Structure et ultrastructure des cellules mesenchymateuses et nerveuses intramesogleennes de *Veretillium cynomorium* Pall. (Cnidaria, Pennatulidae).
Vie Milieu Ser., A20: 279-292.

Byskov, A.G. (1978)
Follicular atresia. In R.E. Jones (Ed.) "The Vertebrate Ovary, Comparative Biology and Evolution".
New York, Plenum Press, pp. 533-562.

Byskov, A.G. (1979)
Atresia. In A.R. Midgely and W.A. Sadler (Eds.) "Ovarian Follicular Development and Function".
New York, Raven Press, pp. 41-57.

Cain, A.J. (1974)
Breeding system of a sessile animal.
Nature, 247: 289-290.

Callen, J.C., Dennebouy, N. and Mounolou, J.C. (1980)
Development of the mitochondrial mass and accumulation of mtDNA in previtellogenic stages of *Xenopus laevis* oocytes. J. Cell Sci., 41: 307-320.

Calow, P. (1984)
Exploring the acaptive landscapes of invertebrate life cycles. In W. Engels (Ed.) "Advances in Invertebrate Reproduction 3". Amsterdam, Elsevier, pp. 329-342.

Campbell, R.D. (1973)
Vital marking of single cells in developing tissues: india ink injection to trace tissue movement in *Hydra*. J. Cell Sci., 13: 651-661.

Campbell, R.D. (1974a)

Development. In L. Muscatine and H.M. Lenhoff (Eds.) "Coelenterate Biology - Reviews and New Perspectives". New York, Academic Press, pp. 179-210.

Campbell, R.D. (1974b)

Cnidaria. In A.C. Giese and J.S. Pearse (Eds.) "Reproduction of Marine Invertebrates, Vol. 1". New York, Academic Press, pp. 133-199.

Carter, M.A. and Funnell, M.E. (1980)

Reproduction and brooding in *Actinia*. In P. Tardent and R. Tardent (Eds.) "Developmental and Cellular Biology of Coelenterates". Amsterdam, Elsevier/ North Holland Biomedical Press, pp 17-22.

Carter, M.A. and Thorp, C.H. (1979)

The reproduction of *Actinia equina* L. var. *mesembryanthemum*.

J. mar. biol. Ass. UK., 59: 989-1001.

Carter, M.A. and Thorpe, J.P. (1981)

Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific.

J. mar. biol. Ass. UK., 61: 79-93.

Chapman, D.M. (1974)

Cnidarian histology. In L. Muscatine and H.M. Lenhoff (Eds.) "Coelenterate Biology - Reviews and New Perspectives". New York, Academic Press, pp. 2-92.

Chapman, D.M. and James, R. (1973)

Intraepithelial flagella in the medusa of *Aurelia aurita* (L).

Publ. Seto Mar. Biol. Lab., 20: 731-743.

Chia, F.S. and Crawford, B.J. (1973)
Some observations on gametogenesis, larval development, and substratum selection of the sea pen *Ptilosarcus gurneyi*. Mar. Biol., 23: 73-82.

Chia, F.S. and Crawford, B.J. (1977)
Comparative fine structural studies of planulae and primary polyps of identical age of the sea pen *Ptilosarcus gurneyi*. J. Morphol., 151: 131-158.

Chia, F.S. and Koss, R. (1979)
Fine structural studies of the nervous system and the apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*.
J. Morphol., 160: 275-298.

Chia, F.S. and Rostron, M.A. (1970)
Some aspects of the reproductive biology of *Actinia equina* (Cnidaria: Anthozoa).
J. mar. biol. Ass. UK., 50: 253-264.

Chia, F.S. and Spaulding, J.G. (1972)
Development and juvenile growth of the sea anemone *Tealia crassicornis*. Biol. Bull., 142: 206-218.

Clark, W.H. and Dewel, W.C. (1974)
The structure of the gonads, gametogenesis and sperm-egg interactions in the Anthozoa.
Amer. Zool., 14: 495-510.

Colley, N.J. and Trench, R.K. (1983)
Selectivity in phagocytosis and persistence of symbiotic algae by the scyphistoma stage of the jellyfish *Cassiopeia xamachana*.
Proc. R. Soc. Lond., B219: 61-82.

Cormier, S.M. and Hessinger, D.A. (1980)
Cnidocil apparatus: sensory receptor of *Physalia* nematocytes. J. Ultrastruct. Res., 72: 13-19.

Cowden, R.R. (1964)
A cytochemical study of gonophore and oocyte development in *Pennaria tiarella*.
Act. Emb. Morphol. Exp., 7: 167-179.

Dale, B. and Monroy, A. (1981)
How is polyspermy prevented?
Gamete Res., 4: 151-169.

Dales, R.P. and Dixon, L.R.J. (1981)
Polychaetes. In N.A. Ratcliffe and A.F. Rowley (Eds.) "Invertebrate Blood Cells, Vol. 1". London, Academic Press, pp. 35-74.

David, C.N. (1973)
A quantitative method for the maceration of *Hydra* tissue.
Wilhelm Roux. Arch., 171: 259-268.

David, C.N. (1983)
Stem cell proliferation and differentiation in *Hydra*. In C.S. Potten (Ed.) "Stem Cells - Their Identification and Characteristics". New York, Churchill Livingstone Chapter 2.

David, C.N. and Murphy, S. (1977)
Characterization of interstitial stem cells in *Hydra* by cloning. Dev. Biol., 58: 372-383.

De Bruyn, P.P.H., Cho, Y. and Michelson, S. (1983)
In vivo endocytosis by bristle coated pits of protein tracers and their intracellular transport in the endothelial cells lining the sinuses of the liver.

1. The endosomal disposition.

J. Ultrastruct. Res., 85: 272-289.

De Loof, A. (1984)

Evolution and hormones controlling reproduction.

In W. Engels (Ed) "Advances in Invertebrate

Reproduction 3". Amsterdam. Elsevier, pp. 139-149.

De Robertis E.D.P., Saez, F.A. and De Robertis, E.M.F. (1975)

"Cell Biology"(6th Edition). Philadelphia,

W.B. Saunders Company.

De Vos, L. (1971)

Etude ultrastructurale de la gemmulogenese chez

Ephydatia fluviatilis. 1. Le vitellus - formation

teneur en ARN et glucogene.

J. Microscopie, 10: 283-304.

Dewel, W.C. and Clark, W.H. (1972)

An ultrastructural investigation of spermiogenesis

and the mature sperm in the anthozoan *Bunodosoma*

cavernata (Cnidaria).

J. Ultrastruct. Res., 40: 417-431.

Dewel, W.C. and Clark, W.H. (1974)

A fine structural investigation of surface

specializations and the cortical reaction in eggs

of the cnidarian *Bunodosoma cavernata*.

J. Cell Biol., 60: 78-91.

Dhainaut, A. (1969)

Origine et structure des formations mucopoly-

saccaridiques de la zone corticale de l'ovocyte de

Nereis diversicolor O.F. Müller (Annelide Polychete)

J. Microscopie, 8: 69-86.

Dhainaut, A., Porchet, M., Fischer, A. and Baert, J-L. (1984)
Biochemical and metabolic aspects of oocyte differentiation in nereids (Annelida, Polychaeta). In W. Engels (Ed.) "Advances in Invertebrate Reproduction 3." Amsterdam, Elsevier, pp. 3-16.

Diaz, J-P. (1979)
La degenerescence ovocytaire chez la demosponge *Suberites massa*. In C.Levi and N. Boury-Esnault (Eds,) "Biologie des Spongiaires". Colloques Internationaux du CNRS., no. 291: 79-86.

Doumenc, D.A. (1977)
Etude dynamique de la morphogénèse au cours des phases actinella et edwardsia de l'actinie *Cereus pedunculatus* Pennant.
Arch. Zool. Exp. Gen., 118: 79-102.

Dumont, J.N. and Anderson, E. (1967)
Vitellogenesis in the horseshoe crab, *Limulus polyphemus*. J. Microscopie, 6: 791-806.

Dunn, D.F. (1975)
Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill.
Biol. Bull., 148: 199-218.

Dunn, D.F. (1982)
Sexual reproduction of two intertidal sea anemones (Coelenterata: Actinaria) in Malaysia.
Biotropica, 14: 262-271.

Dunn, D.F., Chia, F.S. and Levine, R. (1980)
Nomenclature of *Aulactinia (=Bunodactis)* with description of *Aulactinia incubans* n.sp. (Coelenterata: Actinaria), an internally brooding sea anemone from

Puget Sound.

Can. J. Zool., 58: 2071-2080.

Dym, M. and Fawcett, D.W. (1971)

Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis.

Biol Reprod., 4: 195-215.

Eckelbarger, K.J. (1979)

Ultrastructural evidence for both autosynthetic and heterosynthetic yolk formation in the oocytes of an annelid *Phragmatopoma lapidosa* (Polychaeta).

Tissue and Cell, 11: 425-443.

Eckelbarger, K.J. (1980)

An ultrastructural study of oogenesis in *Streblospio benedicti* (Spionidae), with remarks on diversity of vitellogenic mechanisms in Polychaeta.

Zoomorphology, 94: 241-263.

Eckelbarger, K.J. (1983)

Evolutionary radiation in polychaete ovaries and vitellogenic mechanisms: their possible role in life history patterns.

Can. J. Zool., 61: 487-504.

Eckelbarger, K.J. and Grassle, J.P. (1982)

Ultrastructure of the ovary and oogenesis in the polychaete *Capitella jonesi* (Hartman, 1959).

J. Morphol. 171: 305-320.

Eckelbarger, K.J., Linley, P.A. and Grassle, J.P. (1984)

Role of ovarian follicle cells in vitellogenesis and oocyte resorption in *Capitella* sp. 1. (Polychaeta).

Mar. Biol., 79: 133-144.

Eddy, E.M. (1975)
Germ plasm and the differentiation of the germ cell line. Int. Rev. Cytol., 43: 229-280.

Eddy, E.M., Clark, J.M., Gong, D. and Fenderson, B.A. (1981)
Origin and migration of primordial germ cells in mammals. Gamete Res., 4: 333-362.

Epel, D. (1967)
Protein synthesis in sea urchin egg: A 'late' response to fertilization.
Proc. Natl. Acad. Sci. USA., 57: 889-906.

Epel, D. (1975)
The program and mechanisms of fertilization in the echinoderm egg. Am. Zool., 15: 507-522.

Fadlallah, Y.H. and Pearse, J.S. (1982a)
Sexual reproduction in solitary corals: overlapping oogenic and brooding cycles, and benthic planulas in *Balanophyllia elegans*. Mar. Biol., 71: 223-231.

Fadlallah, Y.H. and Pearse, J.S. (1982b)
Sexual reproduction in solitary corals: synchronous gametogenesis and broadcast spawning in *Paracyathus stearnsii*. Mar. Biol., 71: 233-239.

Fain-Maurel, M.A. (1968)
Variabilité de la structure mitochondriale dans les mucocytes des glandes salivaires de *Limnea stagnalis* L. (Gastropode Pulmoné).
C.r. Hebd. Seance. Acad. Sci., 267: 1614-1616.

Fallon, J. and Austin, C.R. (1967)
Fine structure of gametes of *Nereis limbata* (Annelida) before and after interaction.
J. Exp. Zool., 166: 225-242.

Fawcett, D.W., Anderson, W.H. and Phillips, D.M. (1971)
Morphogenetic factors influencing the shape of the sperm head. Dev. Biol., 26: 220-251.

Fell, P.E. (1969)
The involvement of nurse cells in oogenesis and embryonic development in the marine sponge *Haliclona ecbasis*. J. Morphol., 127: 133-150.

Franc, S. (1970)
Les evolutions cellulaires au cours de la regeneration du pedoncule de *Veretillium cynomorium* Pall.
Vie Milieu Ser., A21: 49-93.

Franzen, A. (1956)
On spermiogenesis, morphology of the spermatozoon, and biology of fertilization among invertebrates.
Zool. Bidrag. Upps., 30: 355-482.

Franzen, A. (1970)
Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. In B. Baccetti (Ed.) "Comparative Spermatology". New York, Academic Press, pp. 29-46.

Franzen, A. (1977)
Sperm structure with regard to fertilization biology and phylogenetics.
Verh. Dtsch. Zool. Ges., pp. 123-138.

Fujiwara, K. and Pollard, T.D. (1976)
Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells. J. Cell Biol., 71: 848-875.

Gallissian, M-F. and Vacelet, J. (1976)
Ultrastructure de quelques stades de l'ovogenèse de spongiaires du genre *Verongia*(Dictoceratida).
Ann. Sci. Nat. Zool., Paris, 18: 381-404.

Gashout, S.E. and Ormond, R.F.G. (1979)
Evidence for parthenogenetic reproduction in the sea anemone *Actinia equina*.
J. mar. biol. Ass. UK., 59: 975-987.

Gemmill, J.F. (1920)
The development of the sea anemones *Metridium dianthus* (Ellis) and *Adamsia palliata* (Bohad).
Phil. Trans. R. Soc. Lond., B209: 351-375.

Gemmill, J.F. (1921)
The development of the sea anemone *Bolocera tuediae* (Johnst.).
Quart. J. Microscop. Sci., 65: 577-587.

Geuze, M.J., Slot, J.W., Straus, J.A.M., Peppard, J., Von Figura, K., Hasilik, A. and Schwartz, A.L. (1984)
Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. Cell, 37: 195-204.

Gierer, A. (1977)
Biological features and physical concepts of pattern formation exemplified by hydra.
Curr. Top. Dev. Biol., 11: 17-59.

Gierer, A. and Meinhardt, H. (1972)
A theory of biological pattern formation.
Kybernetik, 12: 30-39.

Glabe, C.G. and Vacquier, V.D. (1978)
Egg surface glycoprotein receptor for sea urchin sperm binding.
Proc. Natl. Acad. Sci. USA., 75: 881-885.

Glatzer, K.H. (1971)
Die Ei- und Embryonalentwicklung von *Corydendrium parasiticum* mit besonderer Berucksichtigung der Oocyten-Feinstruktur während der Vitellogenese.
Helgoland. Wiss. Meeresunters., 22: 213-280.

Goldfischer, S. (1982)
The internal reticular apparatus of Camillo Golgi: a complex, heterogeneous organelle, enriched in acid, neutral and alkaline phosphatases, and involved in glycosylation, secretion, membrane flow, lysosome formation and intracellular digestion.
J. Histochem. Cytochem., 30: 717-733.

Gould-Somero, M. and Holland, L. (1975)
Fine structural investigation of the insemination response in *Urechis caupo*.
Dev. Biol., 46: 358-369.

Graham, R.C. and Karnovsky, M.J. (1966)
The early stages of absorption of injected horse-radish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique
J. Histochem. Cytochem., 14: 291-302.

Grimstone, A.V., Horne, R.W., Pantin, C.F.A. and Robson, E.A. (1958)
The fine structure of the mesenteries of the sea anemone *Metridium senile*.
Quart. J. Microscop. Sci., 99: 623-540.

Guraya, S.S. (1982)
Recent progress in the structure, origin, composition and function of cortical granules in animal egg.
Int. Rev. Cytol., 78: 257-360.

Hand, A.R. and Oliver, C. (1977)
Cytochemical studies of GERL and its role in secretory granule formation in exocrine cells.
Histochem. J., 9: 375-392.

Hanker, J.S., Yates, P.E., Metz, C.B. and Rustioni, A. (1977)
A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase.
Histochem. J., 9: 789-792.

Hamisch, J. (1971)
Die Blastostyle - und Spermienentwicklung von *Eudendrium racemosum* Cavolini.
Zool. Jb. Anat., 87: 1-62.

Harrison, J.A. (1966)
Some observations on the presence of annulate lamellae in alligator and sea gull adrenal cortical cells. J. Ultrastruct. Res., 14: 158-166.

Helenius, A., Mellman, I., Wall, D and Hubbard, A. (1983)
Endosomes
Trends Biochem. Sci., 7: 245-250.

Hertwig, O. and Hertwig, R. (1879)
"Die Actinien." Jena: Verlag von Gustav Fischer,
224 pp.

Heuser, J.E. and Kirschner, M.W. (1980)
Filament organization resolved in platinum replicas of freeze-dried cytoskeletons.
J. Cell Biol., 86: 212-234.

Hill, R.S. and Bowen, I.D. (1976)
Studies on the ovotestis of the slug *Agriolimax reticulatus* (Muller). 1. The oocyte.
Cell Tiss. Res., 173: 465-482.

Hinsch, G.W. (1974)
Comparative ultrastructure of cnidarian sperm.
Am. Zool., 14: 457-465.

Hinsch, G.W. and Clark, W.H. (1973)
Comparative fine structure of cnidarian spermatozoa.
Biol. Reprod., 8: 62-73.

Hirokawa, N. and Heuser, J.E. (1981)
Quick-freeze, deep-etch visualization of the cytoskeleton beneath surface differentiations of intestinal epithelial cells.
J. Cell Biol., 91: 399-409.

Hirokawa, N. and Tilney, L.G. (1982)
Interactions between actin filaments, and actin filaments and membranes, in quick-frozen and deeply etched hair cells of the chick ear.
J. Cell Biol., 95: 249-261.

Hirokawa, N., Tilney, L.G., Fujiwara, K. and Heuser, J.E. (1982)
The organisation of actin, myosin and intermediate filaments in the brush border of intestinal epithelial cells. J. Cell Biol., 94: 425-443.

Holley, M.C. (1982)
Control of anthozoan cilia by the basal apparatus.
Tissue and Cell, 14: 607-620

Holley, M.C. (1983)
The function of the basal apparatus in anthozoan cilia. J. Submicrosc. Cytol., 15: 127-132.

Holley, M.C. (1984)
The ciliary basal apparatus is adapted to the structure and mechanics of the epithellium.
Tissue and Cell, 16: 287-310.

Honegger, T.G. (1980)
Gametogenesis of *Sertularella polyzonias* with special reference to vitellogenesis (Hydrozoa, Athecata). In P. Tardent and R.Tardent (Eds:) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 67-73.

Honegger, T.G. (1983)
Ultrastructural and experimental investigations of sperm-egg interactions in fertilization of *Hydra carnea*.
Roux's Arch. Dev. Biol., 192: 13-20.

Hruban, Z., Swift, H. and Slesers, A. (1965).
Effect of azaserine on the fine structure of the liver and pancreatic acinar cells.
Cancer Res., 25: 708-723.

Humphreys, W.J. (1967)
The fine structure of cortical granules in eggs and gastrulae of *Mytilus edulis*.
J. Ultrastruct. Res., 17: 314-326.

Hyman, L.H. (1940)
"The Intertebrates: Protozoa through Ctenophora. The Acoelomate Bilataria" New York, McGraw-Hill.

Illmensee, K. and Mahowald, A.P. (1974)
Transplantation of posterior polar plasma in *Drosophila*. Induction of germ cells at the anterior pole of the egg.
Proc. Natl. Acad. Sci. USA., 71: 1016-1020.

Illmensee, K. and Mahowald, A.P. (1976)
The autonomous function of germ plasm in a somatic region of the *Drosophila* egg.
Exp. Cell Res., 97: 127-140.

Ishida, J. (1936)
Digestive enzymes of *Actinia mesembryanthemum*.
Annotnes. Zool. Jap., 15: 285-305.

Jennison, B.L. (1978)
Effects of thermal effluents on reproduction in a sea anemone. In J.H. Thorp and J.W. Gibbons (Eds.) "Energy and Environmental Stress in Aquatic Systems" US Dept. Energy Conf., 77114: 470-483.

Jennison, B.L. (1979)
Gametogenesis and reproductive cycles in the sea anemone *Anthopleura elegantissima* (Brandt, 1835).
Can. J. Zool., 57: 403-411.

Jennison, B.L. (1981)
Reproduction in three species of sea anemones from Key West, Florida.
Can. J. Zool., 59: 1708-1719.

Johnston, M.A., Elder, H.Y. and Spencer-Davies, P. (1973)
Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism.
Comp. Biochem. Physiol., 46A: 569-581.

Kawaguti, S. and Ogasawara, Y. (1967)
Electron microscopy on the ovary of an Anthomedusa, *Spirocodon saltatrix*.
Biol. J. Okayama Univ., 13: 115-129.

Keilin, D. and Hartree, E.F. (1951)
Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin. Biochem. J., 49: 88-104.

Kerr, J.B. and Dixon, K.E. (1974)
An ultrastructural study of germ plasm in spermato-

genesis of *Xenopus laevis* .
J. Embryol. exp. Morph., 32: 573-592.

Kerr, J.F.R. (1971)
Shrinkage necrosis: a distinct mode of cellular death. J. Path., 105: 13-20.

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972)
Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.
Br. J. Cancer, 26: 239-257.

Kessel, R.G. (1968a)
Electron microscope studies on developing oocytes of a coelenterate medusa with special reference to vitellogenesis. J. Morphol., 126: 211-248.

Kessel, R.G. (1968b)
An electron microscope study of differentiation and growth in oocytes of *Ophioderma panamensis*.
J. Ultrastruct. Res., 22: 63-89.

Kessel, R.G. (1968c)
Annulate lamellae.
J. Ultrastruct. Res., Suppl. 10: 3-82.

Kevin, M.J., Hall, W.T., McLaughlin, J.J.A. and Zahl, P.A. (1969)
Symbiodinium microadriaticum Freudenthal, a revised taxonomic description, ultrastructure.
J. Phycol., 5: 341-350.

King, R.F. (1983)
The organization of actin filaments in human placental villi.
J. Ultrastruct. Res., 85: 320-328.

Kinnamon, J.C. and Westfall, J.A. (1984)
High voltage electron stereomicroscopy of the cilium-stereociliary complex of perioral sensory cells in *Hydra*.
Tissue and Cell, 16 4 345-353.

Kleve, M.G. and Clark, W.H. (1976)
The structure and function of centriolar satellites and pericentriolar processes in Cnidarian sperm.
In G.O. Mackie (Ed.) "Coelenterate Ecology and Behaviour". New York, Plenum Press, pp. 309-317.

Krigsman, B.J. and Talbot, F.H. (1953)
Experiments on digestion in sea anemones.
Archs. Int. Physiol., 61: 277-291.

Larkman, A.U. (1980)
Ultrastructural aspects of gametogenesis in *Actinia equina* L. In P. Tardent and R. Tardent (Eds) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 61-66.

Larkman, A.U. (1981)
An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria; Anthozoa).
Int. J. Invertbr. Reprod., 4: 147-167.

Larkman, A.U. (1983)
An ultrastructural study of oocyte growth within the endoderm and entry into the mesogloea in *Actinia fragacea* (Cnidaria; Anthozoa).
J. Morphol., 178: 155-177.

Larkman, A.U. (1984a)
An ultrastructural study of the establishment of the testicular cysts during spermatogenesis in the sea anemone *Actinia fragacea* (Cnidaria; Anthozoa). Gamete. Res., 9: 303-327.

Larkman, A.U. (1984b)
The fine structure of mitochondria and the mitochondrial cloud during oogenesis in the sea anemone *Actinia*. Tissue and Cell, 16: 393-404.

Larkman, A.U. (1984c)
The fine structure of granular amoebocytes from the gonads of the sea anemone *Actinia fragacea* (Cnidaria; Anthozoa). Protoplasma, 122: 203-221.

Larkman, A.U. and Carter, M.A. (1980)
The spermatozoon of *Actinia equina* L. var. *mesembryanthemum*. J. mar. biol. Ass. UK., 60: 193-204.

Larkman, A.U. and Carter, M.A. (1982)
Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a nutritive function. Int. J. Invertebr. Reprod., 4: 375-379.

Larkman, A.U. and Carter, M.A. (1984)
The apparent absence of a cortical reaction after fertilization in a sea anemone. Tissue and Cell, 16: 125-130.

Lenhoff, H.M. (1980)
Our link with the Trembleys - Abraham (1710-1784), Maurice (1874-1942) and Jean-Gustave (1903-1977). In P. Tardent and R. Tardent (Eds.) "Developmental

and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. XVII to XXIV.

Lenhoff, H.M. and Brown, R.D. (1970)
Mass culture of *Hydra*: an improved method and its application to other aquatic invertebrates.
Lab. Animals, 4: 139-154.

Lentz, T.L. (1966)
"The Cell Biology of Hydra" Amsterdam, North Holland Publishing Co..

Lewis, C.A., Talbot, C.F. and Vacquier, V.D. (1982)
A protein from abalone sperm dissolves the egg vitelline layer by a non-enzymatic mechanism.
Dev. Biol., 92: 227-239.

Lewis, D.H. and Smith, D.C. (1971)
The autotrophic nutrition of symbiotic marine coelenterates with special reference to hermatypic corals. 1. Movement of photosynthetic products between the symbionts.
Proc. R. Soc. Lond., B178: 111-129.

Lewis, P.R. and Knight, D.P. (1977)
"Staining Methods for Sectioned Material" Amsterdam, North Holland Publishing Co..

Littlefield, C.L. (1984)
Evidence for a germline in *Hydra oligactis* males.
In W. Engels (Ed.) "Advances in Invertebrate Reproduction 3" Amsterdam, Elsevier Science Publishers, p. 208.

Longo, F.J. (1976)

Cortical changes in *S. isula* eggs upon insemination.

J. Ultrastruct. Res., 56: 226-232.

Loseva, L.M. (1971a)

Observations on oogenesis of actinians. 1. Oogenesis in *Bunodactis stella*.

Vestn. Leningr. Univ. Biol., 25: 49-61.

Loseva, L.M. (1971b)

Observations on oogenesis of actinians. II. Oogenesis in *Tealia crassicornis* (Muell), *Metridium senile* (L.) and *Protanthea simplex*.

Vestn. Leningr. Univ. Biol., 26: 22-29.

Lyke, E.B. and Robson, E.A. (1975)

Spermatogenesis in Anthozoa: differentiation of the spermatid. Cell Tiss. Res., 157: 185-205.

Lyons, K.M. (1973)

Collar cells in planula and adult ectoderm of the solitary coral *Balanophyllia regia*.

Z. Zellforsch., 145: 57-74.

Mabuchi, I. and Okuno, M. (1977)

The effect of myosin antibody on the division of starfish blastomeres.

J. Cell Biol., 74: 251-263.

Mahowald, A.P. (1962)

Fine structure of pole cells and polar granules in *Drosophila melanogaster*.

J. Exp. Zool., 151: 201-215.

Mahowald, A.P., Allis, C.D., Karrer, K.M., Underwood, E.M. and Waring, G.L. (1979)

Germ plasm and pole cells in *Drosophila*. In S. Subtelny and I.R. Konigsberg (Eds.) "Determinants of Spatial Organization" New York, Academic Press, pp. 127-146.

Mahowald, A.P. and Boswell, R.E. (1983)

Germ plasm and germ cell development in invertebrates. In A. McLaren and C.C. Wylie (Eds.) "Current Problems in Germ Cell Differentiation" Cambridge, Cambridge University Press, pp. 3-17.

Mahowald, A.P. and Hennen, S. (1971)

Ultrastructure of the 'germ plasm' in eggs and embryos of *Rana pipiens*. Dev. Biol., 24: 37-53.

Marcum, B.A. and Campbell, R.D. (1978)

Development of *Hydra* lacking nerve and interstitial cells. J. Cell Sci., 29: 17-33.

Maul, G.G. (1977)

The nuclear and the cytoplasmic pore complex: structure, dynamics, distribution and evolution. Int. Rev. Cytol., Suppl 6: 75-186.

McCulloch, D. (1952)

Fibrous structures in the ground cytoplasm of the *Arbacia* egg. J. Exp. Zool., 119: 47-58.

Mergner, H. (1971)

Cnidaria. In G. Reverberi (Ed.) "Experimental Embryology of Marine and Fresh Water Invertebrates" Amsterdam, North Holland Publishing Co., pp. 1-84.

Miller, R.L. (1983)
Cnidaria. In K.G. Adiyodi and R.G. Adiyodi (Eds.) "Reproductive Biology of Invertebrates. Volume II. Spermatogenesis and Sperm Function" London, John Wiley; Chapter 2.

Millonig, G., Bosco, M. and Giambertone, L. (1969)
Fine structure analysis of oogenesis in sea urchins. J. Exp. Zool., 169: 293-314.

Minasian, L.L. (1980)
The distribution of proliferating cells in an anthozoan polyp, *Haliplanella luciae* (Actiniaria; Acontiaria), as indicated by ^{3}H-thymidine incorporation. In P. Tardent and R. Tardent (Eds.) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 415-420.

Mooseker, M.S. (1976)
Brush border motility: microvillar contraction in Triton-treated brush borders isolated from intestinal epithelium. J. Cell Biol., 71: 417-433.

Mooseker, M.S., Keller, T.C.S. and Hirokawa, N. (1983)
Regulation of cytoskeletal structure and contractility in the brush border. In R. Porter and G.M. Collins (Eds.) "Brush Border Membranes" London, Pitman Books Ltd., pp. 195-215.

Morales, R. and Duncan, D. (1971)
Prismatic and other unusual arrays of mitochondrial cristae in astrocytes of cats and hamsters. Anat. Rec., 171: 545-558.

Murdock, L., Cahill, M.A. and Reith, A. (1977)
Morphometry and ultrastructure of prismatic cristae in mitochondria of a crayfish muscle. A hypothesis of the structural principle.
J. Cell Biol., 74: 326-332.

Muscatine, L. and Cernichiari, E. (1969)
Assimilation of photosynthetic products of zooxanthellae by a reef coral.
Biol. Bull., 137: 506-523.

Needham, J. (1931)
"Chemical Emrbyology. Vol. 1" New York, Hafner Publishing Company, pp. 44-227.

Nicol, J.A.C. (1959)
Digestion in sea anemones.
J. mar. biol. Ass. UK., 38: 469-477.

Nieuwkoop, P.D. and Satasurya, L.A. (1979)
"Primordial Germ Cells in the Chordates" Cambridge, Cambridge University Press, 187 pp.

Nieuwkoop, P.D. and Satasurya, L.A. (1981)
"Primordial Germ Cells in the Invertebrates"
Cambridge, Cambridge University Press, 258 pp.

Noda, K. and Kanai, C. (1977)
An ultrastructural observation on *Pelmatohydra robusta* at sexual and asexual stages, with a special reference to 'germinal plasm'.
J. Ultrastruct. Res., 61: 284-294.

Noda, K. and Kanai, C. (1980)
An ultrastructural observation on the embryogenesis of *Pelmatohydra robusta* with special reference to 'germinal dense bodies'. In P. Tardent and R. Tardent

(Eds). "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 133-138.

Norrevang, A. (1968)
Electron microscopic morphology of oogenesis.
Int. Rev. Cytol., 23: 113-186.

Nuccitelli, R. and Grey, R.D. (1984)
Controversy over the fast, partial, temporary block to polyspermy in sea urchins: a re-evaluation.
Dev. Biol., 103: 1-17.

Nussbaum, M. (1880)
Zur Differenzierung des Geschlechts im Thierreich.
Arch. Mikrosk. Anat., 18: 1-121.

Nyholm, K.G. (1943)
Zur Entwicklung und Entwicklungs-biologie der Ceriantharien und Aktinien.
Zool. Bidr. Uppsala, 22: 87-248.

Nyholm, K.G. (1949)
On the development and dispersal of athenaria actinia with special reference to *Halcampa duodecimcirrata* M. Sars.
Zool. Bidr. Uppsala, 27: 465-506.

O'Donnell, M.J. and Maddrell, S.H.P. (1983)
Paracellular and transcellular routes for water and solute movements across insect epithelia.
J. Exp. Biol., 106: 231-253.

Okada, M., Kleinman, I.A. and Schneiderman, H.A. (1974)
Restoration of fertility in sterilized *Drosophila* eggs by transplantation of polar cytoplasm.
Dev. Biol., 37: 43-54.

Olive, P.J.W., Garwood, P.R. and Bentley, M.G. (1981)
Reproductive failure and oosorption in Polychaeta in relation to their reproductive strategies.
Bull. Soc. Zool. Fr., 106: 263-268.

Orr, J., Thorpe, J.P. and Carter, M.A. (1982)
Biochemical and genetic confirmation of the asexual reproduction of brooded offspring in the sea anemone *Actinia equina*.
Mar. Ecol. Prog. Ser., 7: 227-229.

Ottaway, J.R. (1980)
Population ecology of the intertidal anemone *Actinia tenebrosa*. IV. Growth rates and longevities.
Aust. J. Mar. Freshwater Res., 31: 385-395.

Paglia, L.M., Berry, S.J. and Kastern, W.H. (1976)
Messenger RNA synthesis, transport and storage in silkmoth ovarian follicles.
Dev. Biol., 51: 173-181.

Parsons, D.S. (1983)
Introductory remarks on the brush border. In R.Porter and G.M. Collins (Eds.) "Brush Border Membranes"
London, Pitman Books Ltd., pp. 3-11.

Pastan, I. H. and Willingham, M.C. (1981)
Receptor-mediated endocytosis of hormones in cultured cells. Ann. Rev. Physiol., 43: 239-250.

Patterson, M.J. and Landolt, M.L. (1979)
Cellular reaction to injury in the anthozoan *Anthopleura elegantissima*.
J. Invert. Pathol., 33: 189-196.

Paul, M. (1975)
The polyspermy block in eggs of *Urechis caupo*.
Expl. Cell Res., 90: 137-142.

Pearse, B.M.F. and Bretscher, M.S. (1981)
Membrane recycling by coated vesicles.
Ann. Rev. Biochem., 50: 85-101.

Peluso, J.J. (1979)
In vitro maturation of oocytes collected from atretic follicles. In A.R. Midgely and W.A. Sadler (Eds.) "Ovarian Follicular Development and Function" New York, Raven Press, pp. 85-88.

Peters, H. and McNatty, K.P. (1980)
"The Ovary" London, Paul Elek, Chapter 8, pp 98-106.

Peteya, D.J. (1975)
The ciliary-cone sensory cells of anemones and cerianthids. Tissue and Cell, 7: 243-252.

Raabe, M. (1984)
Insect neurohormones & female reproduction.
In W. Engels (Ed.) "Advances in Invertebrate Reproduction 3" Amsterdam, Elsevier, pp. 163-176.

Rash, J.E., Ahay, J.W. and Biesele, J.J. (1969)
Cilia in cardiac differentiation.
J. Ultrastruct. Res., 29: 470-484.

Rebhun, L.I. (1961)
Some electron microscope observations on membranous basophilic elements of invertebrate eggs.
J. Ultrastruct. Res., 5: 208-

Reynolds, E.S. (1963)

The use of lead citrate at high pH as an electron-opaque stain in electron microscopy.

J. Cell Biol., 17: 208-212.

Riemann-Zurneck, K. (1976)

Reproductive biology, oogenesis and early development in the brood-caring sea anemone *Actinostola spetsbergensis* (Anthozoa: Actiniaria).

Helgoland. Wiss. Meeresunters., 28: 239-249.

Rinkevich, B. and Loya, Y. (1979)

The reproduction of the Red Sea coral *Stylophora pistillata*. 1. Gonads and planulae.

Mar. Ecol. Prog. Ser., 1: 133-144.

Roosen-Runge, E.C. (1977)

"The Process of Spermatogenesis in Animals"

Cambridge, Cambridge University Press, 214 pp.

Roosen-Runge, E.C. and Szollosi, D. (1965)

On biology and structure of the testis of *Phialidium* Leuckhart (Leptomedusae).

Z. Zellforsch., 68: 597-610.

Rossi, L. (1974)

Sexual races in *Cereus pedunculatus*.

Pubbl. Staz. Zool. Napoli, 39: 463-471.

Rostron, M.A. (1970)

"The Reproductive Biology of the Viviparous Sea Anemone *Actinia equina* L."

PhD Thesis, University of London.

Rostron, M.A. and Rostron, J. (1978)

Fecundity and reproductive ecology of a natural population of *Actinia equina* L. (Cnidaria: Anthozoa).

J. exp. mar. Biol. Ecol., 33: 251-259.

Runnström, J. (1966)

The vitelline membrane and cortical particles in sea urchin eggs and their function in maturation and fertilization.

Adv. Morphogenesis, 5: 221-325.

Schade, M.L. and Shivers, R.R. (1980)

Structural modulation of the surface and cytoplasm of oocytes during vitellogenesis in the lobster *Homarus americanus*. An electron microscope-protein tracer study. J. Morphol., 163: 13-26.

Schäfer, W. (1981)

Fortpflanzung und Sexualitat von *Cereus pedunculatus* und *Actinia equina* (Anthozoa, Actiniaria).

Helgoland. Wiss. Meeresunter., 34: 451-461.

Schäfer, W. (1983)

Verleichende Untersuchungen uber Struktur und Genese der Anthozoenoocyten. Teil 1; Fruhentwicklung und innere Differenzierung der Oocyten.

Zool. Jb. Anat., 109: 407-448.

Schäfer, W.G. and Schmidt, H. (1980)

The anthozoan egg: differentiation of internal oocyte structure. In P. Tardent and R. Tardent (Eds.) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 47-51.

Schechtman, A.M. (1955)

Ontogeny of the blood and related antigens and their significance for the theory of differentiation.

In E.G. Butler (Ed.) "Biological Specificity and Growth" Princeton, Princeton Univ. Press, pp. 3-31.

Schincariol, A.L. and Habowsky, J.E.J. (1972)
Germinal differentiation of the stem cell in *Hydra fusca*: a model system.
Can. J. Zool., 50: 5-12.

Schlichter, D. (1975)
The importance of dissolved organic compounds in sea water for the nutrition of *Anemonia sulcata* Pennant (Coelenterata). In H. Barnes (Ed.) Proceedings of the 9th Marine Biological Symposium, 1975. Aberdeen, Aberdeen University Press, pp. 395-405.

Schlichter, D. (1978)
On the ability of *Anemonia sulcata* (Coelenterata; Anthozoa) to absorb charged and neutral amino acids simultaneously. Mar. Biol., 45: 97-104.

Schmidt, H. and Holtken, B. (1980)
Peculiarities of spermatogenesis and sperm in Anthozoa. In P. Tardent and R. Tardent (Eds.) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 53-59.

Schmidt, H. and Schäfer, W.G. (1980)
The anthozoan egg: trophic mechanisms and oocyte surfaces. In P. Tardent and R. Tardent (Eds.) "Developmental and Cellular Biology of Coelenterates" Amsterdam, Elsevier/North Holland Biomedical Press, pp. 41-46.

Schmidt, H. and Zissler, D. (1979)
Die Spermien der Anthozoa und ihre phylogenetische Bedeutung.
Zoologica (Stuttg.), 44(129): 1-98.

Schroeder, P.C. (1984)

A summary of the endocrine control of gametogenesis in invertebrates other than insects. In W.Engels (Ed.) "Advances in Invertebrate Reproduction 3" Amsterdam, Elsevier, pp. 203-211.

Schroeder, T.E. (1969)

The role of 'contractile ring' filaments in dividing *Arbacia* egg.

Biol. Bull., 137: 413-414.

Schroeder, T.E. (1973)

Actin in dividing cells: contractile ring filaments bind heavy meromyosin.

Proc. Natl. Acad. Sci. USA., 70: 1688-1692.

Schroeder, T.E. (1982)

Novel surface specialization on a sea anemone egg: 'spires' of actin-filled microvilli.

J. Morphol., 174: 207-216.

Schuel, H. (1978)

Secretory functions of egg cortical granules in fertilization and development: a critical review.

Gamete Res., 1: 299-382.

Shapiro, B.M. and Eddy, E.M. (1980)

When sperm meet egg: biochemical mechanisms of gamete interaction.

Int. Rev. Cytol., 66: 257-302.

Shick, J.M. and Brown, W.I. (1977)

Zooxanthellae-produced O_2 promotes sea anemone expansion and eliminates oxygen debt under environmental hypoxia. J. Exp. Zool., 201: 149-155.

Shick, J.M. and Dykens, J.A. (1984)
Photobiology of the symbiotic sea anemone *Anthopleura elegantissima*: photosynthesis, respiration and behaviour under intertidal conditions.
Biol. Bull., 166: 608-619.

Sica, D., Boniforti, L. and Di Giacomo, G. (1981)
Sterol composition of two Actiniaria.
Comp. Biochem. Physiol., 70B: 153-156.

Siebert, A.E. (1973)
A description of the sea anemone *Stomphia didemon* sp. nov. and its development.
Pac. Sci., 27: 363-376.

Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. (1977)
Endocytosis.
Ann. Rev. Biochem., 46: 669-722.

Singer, I.I. (1971)
Tentacular and oral disc regeneration in the sea anemone *Aiptasia diaphana*. III. Autoradiographic analysis of patterns of tritiated thymidine uptake.
J. Emrbyol. exp. Morphol., 26: 253-270.

Singer, I.I. (1974)
An electron microscopic and autoradiographic study of mesogloeal organization and collagen synthesis in the sea anemone *Aiptasia diaphana*.
Cell Tiss. Res., 149: 537-554.

Slautterback, D.B. (1967)
Coated vesicles in absorptive cells of *Hydra*.
J. Cell Sci., 2: 563-572.

Smith, L.D. (1966)
The role of a 'germinal plasm' in the formation of

primordial germ cells in *Rana pipiens*.
Dev. Biol., 14: 330-347.

Smith, L.D. and Williams, M.A. (1975)
Germinal plasm and determination of the primordial germ cells. In C.L. Marbert and J. Papaconstantinou (Eds.) "The Developmental Biology of Reproduction" New York, Academic Press, pp. 3-24.

Spaulding, J.G. (1972)
The life-cycle of *Peachia quinquecapitata*, an anemone parasitic on medusae during its larval development. Biol. Bull., 143: 440-453,

Spaulding, J.G. (1974)
Embryonic and larval development in sea anemones (Anthozoa; Actiniaria). Am. Zool., 14: 511-520.

Stagni, A. and Lucchi, M.L. (1964)
Ulteriori osservazioni al microscopio elettronico sulla ovogenesi di *Chlorohydra viridissima*.
Rend. Ist. Sci. Camerino., 5: 290-297.

Stagni, A. and Lucchi, M.L. (1970)
Ultrastructural observations on the spermatogenesis in *Hydra attenuata*. In B. Baccetti (Ed.) "Comparative Spermatology" New York, Academic Press, pp. 357-361.

Stein, O. and Stein, Y. (1967)
Lipid synthesis, intracellular transport, storage and secretion. 1. Electron microscopic radioautographic study of liver after injection of tritiated palmitate or glycerol in fasted and ethanol-treated rats. J. Cell Biol., 33: 319-339.

Stephenson, T.A. (1928)
"The British Sea Anemones, Vol. 1" London,
The Ray Society, 148 pp.

Stolte, H.A. (1936)
Die Herkunft des Zellmaterials bei regenerative
Vorgängen der wirbellosen Tiere.
Biol. Rev., 11: 1-49.

Straus, W. (1969)
Use of horseradish peroxidase as a marker protein
for studies of phagolysosomes, permeability and
immunology. In E. Bajusz and G. Jasmin (Eds.)
"Methods and Achievements in Experimental Pathology"
Vol. 4. Basel /New York, Karger, pp. 54-91.

Summers, R.G. and Hylander, B.L. (1975)
Species-specificity of acrosome reaction and
primary gamete binding in echinoids.
Exp. Cell Res., 96: 63-68.

Swift, H. (1956)
The fine structure of annulate lamellae.
J. Biophys. Biochem. Cytol., 2: 415-418.

Szmant-Froelich, A., Yevich, P. and Pilson, M.E.Q. (1980)
Gametogenesis and early development of the temperate
coral *Astrangia danae* (Anthozoa: Scleractinia).
Biol. Bull., 158: 257-269.

Szollosi, D. (1970)
Cortical cytoplasmic filaments of cleaving eggs:
A structural element corresponding to the contractile
ring. J. Cell Biol., 44: 192-209.

Tardent, P. (1954)
Axiale Verteilungs-Gradienten der interstitiellen Zellen bei *Hydra* und *Tubularia* und ihre Bedeutung für die Regeneration.
Wilhelm Roux Arch., 146: 593-649.

Tardent, P. (1974)
Gametogenesis in the genus *Hydra*.
Am. Zool., 14: 447-456.

Tardent, P. and Schmid, V. (1972)
Ultrastructure of mechanoreceptors of the polyp *Coryne pintneri* (Hydrozoa: Athecata).
Exp. Cell Res., 72: 265-275.

Taylor, D.L. (1968)
In situ studies on the cytochemistry and ultrastructure of a symbiotic marine dinoflagellate.
J. mar. biol. Ass. UK., 48: 349-366.

Taylor, D.L. (1969)
The nutritional relationship of *Anemonia sulcata* (Pennant) and its dinoflagellate symbiont.
J. Cell Sci., 4: 751-762.

Taylor, D.L. (1971)
Ultrastructure of the 'zooxanthella' *Eudodinium chattonii, in situ*.
J. mar. biol. Ass. UK., 51: 227-234.

Taylor, G.T. and Anderson, E. (1969)
Cytochemical and fine structural analysis of oogenesis in the gastropod *Ilyanassa obsoleta*.
J. Morphol., 219: 211-247.

Tessenow, W. (1969)
Lytic processes in development of freshwater sponges. In J.T. Dingle and H.B. Fell (Eds.) "Lysosomes in Biology and Pathology" Amsterdam, North Holland, pp. 392-405.

Tiffon, Y. and Dalreaux, M. (1974)
Phagocytose et pinocytose par l'ectoderme et l'endoderme de *Cerianthus lloydii* Gosse.
J. exp. mar. Biol. Ecol., 16: 155-165.

Tourte, M., Mignotte, F. and Mounolou, J-C. (1981)
Organisation and replication activity of the mitochondrial mass of oogonia and previtellogenic oocytes in *Xenopus laevis*.
Develop. Growth and Differ., 23: 9-21.

Trench, R.K. (1971a)
The physiology and biochemsitry of zooxanthellae symbiotic with marine coelenterates. 1. The assimilation of photosynthetic products of zooxanthellae by two marine coelenterates.
Proc. R. Soc. Lond., B177: 225-235

Trench, R.K. (1971b)
The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. II. Liberation of fixed ^{14}C by zooxanthellae *in vitro*.
Proc. R. Soc. Lond., B177: 237-250.

Trench, R.K. (1971c)
The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effect of homogenates of host tissue on the excretion of

photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates.
Proc. R. Soc. Lond., B177: 251-264.

Trump, B.F. and Ginn, F.L. (1969)
The pathogenesis of subcellular reaction to lethal injury. In E. Bajusz and G. Jasmin (Eds.) "Methods and Achievements in Experimental Pathology, Vol. 4" Basel/New York, Karger, pp. 1-29.

Trump, B.F., Goldblatt, P.J. and Stowell, R.E. (1965)
Studies on necrosis of mouse liver *in vitro*. Ultrastructural alterations in the mitochondria of hepatic parenchymal cells.
Lab. Invest., 14: 343-371.

Van der Vyver, G. (1981)
Organisms without special circulatory systems . In N.A. Ratcliffe and A.F. Rowley (Eds) "Invertebrate Blood Cells, Vol. 1" London, Academic Press, pp. 19-32.

Van Praet, M. (1974)
"Regeneration de la region peri-orale d'*Actinia equina*." Thèse de l'Université de Paris, Biologie Animale (Histologie).

Van Praet, M. (1976)
Les activites phosphatasiques acides chez *Actinia equina* L. et *Cereus pedunculatus* P.
Bull. Soc. Zool. Fr., 101: 367-376.

Van Praet, M. (1978)
Etude histochemique et ultrastructurale des zones digestives d'*Actinia equina* L. (Cnidaria: Actinaria).
Cah. Biol. Mar., 19: 415-432.

Van Praet, M. (1980)
Absorption des substances dissoutes dans le milieu, des particules et des produits de la digestion extracellulaire chez *Actinia equina* (Cnidaria: Actiniaria).
Reprod. Nutr. Develop., 20: 1393-1399.

Van Praet, M. and Doumenc, D. (1975)
Morphologie et morphogénèse expérimentale du tentacule chez *Actinia equina*.
J. Microsc. Biol. Cell, 23: 29-38.

Voogt, P.A., Oudejans, R.C.H.M. and Broetjes, J.J.S. (1984)
Steroids and reproduction in starfish. In W. Engels (Ed.) "Advances in Invertebrate Reproduction 3" Amsterdam, Elsevier, pp. 151-161.

Voogt, P.A., Van de Ruit, J.M. and Van Rheenen, J.W.A. (1974)
On the biosynthesis and composition of sterols and sterolesters in some sea anemones.
Comp. Biochem. Physiol., 48B: 225-230.

Vye, M.V. and Fischman, D.A.
The morphological alteration of particulate glycogen by *en bloc* staining with uranyl acetate.
J. Ultrastruct. Res., 33: 278-291.

Walker, C.W. (1974)
Studies on the reproductive system of sea-stars.
1. The morphology and histology of the gonad of

Asterias vulgaris.

Biol. Bull., 147: 661-677.

Walker, C.W. (1980)

Spermatogenic columns, somatic cells, and the microenvironment of germinal cells in the testes of asteroids. J. Morphol., 166: 81-107.

Wallace, R.A. (1978)

Oocyte growth in non-mammalian vertebrates. In R.E. Jones (Ed.) "The Vertebrate Ovary. Comparative Biology - Evolution" New York, Plenum Press, pp. 469-502.

Wallace, R.A. and Dumont, J.N. (1968)

The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*.

J. Cell Physiol., (Suppl.), 72: 73-89.

Wasserthal, W. (1973)

Zur Ei- und Embryonalentwicklung des Hydroidpolypen *Eudendrium armatum*. Eine licht- und elektron-microskopische Untersuchung.

Helgoland. Wiss. Meeresunters., 25: 93-125.

Watson, G.M. and Mariscal, R.N. (1983)

Comparative ultrastructure of catch tentacles and feeding tentacles in the sea anemone *Haliplanella*.

Tissue and Cell, 15: 939-953.

Wedi, S.E. and Dunn, D.F. (1983)

Gametogenesis and reproductive periodicity of the subtidal sea anemone *Urticina lofotensis* (Coelenterata:

Actiniaria) in California.
Biol. Bull., 165: 458-472.

Weismann, A. (1892)
"Das Keimplasma. Eine Theorie der Vererbung."
Jena, Fischer, 628 pp.

Weissman, A., Lentz, T.L. and Barrnett, R.J. (1969)
Fine structural observations on nuclear maturation during spermiogenesis in *Hydra littoralis*.
J. Morphol., 128: 229-240.

West, D.L. (1978)
Ultrastructural and cytochemical aspects of spermiogenesis in *Hydra hymanae*, with reference to factors involved in sperm head shaping.
Dev. Biol., 65: 139-154.

West, D.L. (1980)
Spermiogenesis in the Anthozoan *Aiptasia pallida*.
Tissue and Cell, 12: 243-253.

Westergaard, M. and Von Wettstein, D. (1972)
The synaptinemal complex.
Ann. Rev. Genet., 6: 71-110.

Westfall, J.A. (1966)
The differentiation of nematocytes and associated structures in the Cnidaria.
Z. Zellforsch., 75: 381-403.

Widersten, B. (1965)
Genital organs and fertilization in some Scyphozoa.
Zool. Bidrag. Uppsala, 37: 45-58.

Wischnitzer, S. (1970)
The annulate lamellae. Int. Rev. Cytol., 27: 65-100.

Wolpert, L. (1969)
Positional information and the spatial pattern of cellular differentiation.
J. Theor. Biol., 25: 1-47.

Young, J.A.C. (1974)
The nature of tissue regeneration after wounding in the sea anemone *Calliactis parasitica* (Couch).
J. mar. biol. Ass. UK., 54: 599-617.

Zihler, J. (1972)
Zur Gametogenese und Befruchtungsbiologie von *Hydra*.
Wilhelm. Roux Arch., 169: 239-267.

Addendum

Tiffon, Y. and Hugon, J.S. (1977)
Localisation ultrastructurale de la phosphatase acide et de la phosphatase alcaline dans les cloisons septales stériles de l'anthozoaire *Pachycerianthus fimbriatus*.
Histochemistry, 54: 289-297.

APPENDIX 1

Reagents and procedures used during the processing of tissue for microscopy.

1. Stock solution of 0.2 M phosphate buffer.

0.2 M Na_2HPO_4	76 ml
0.2 M NaH_2PO_4	approx. 24 ml
(add until pH is 7.4)	

2. Glutaraldehyde fixative.

0.2 M phosphate buffer, pH 7.4	50 ml
Glutaraldehyde, EM grade, 25%	12 ml
NaCl	3 g
Distilled water	to make 100 ml

3. Osmium tetroxide fixative.

0.2 M phosphate buffer, pH 7.4	50 ml
Osmium tetroxide crystals	1 g
NaCl	3 g
Distilled water	to make 100 ml

4, Buffer wash solution.

0.2 M phosphate buffer, pH 7.4	50 ml
NaCl	3 g
Distilled water	to make 100 ml

5. 'Emix' epoxy resin is supplied in pre-weighed containers by EMscope Laboratories Limited, Ashford, Kent.

6. Uranyl Acetate Staining Solution.

A saturated solution of uranyl acetate in a 1:1 ethanol/ water mixture.

7. Lead citrate staining solution (after Reynolds, 1963)

Lead nitrate	1.33 g
Sodium citrate	1.76 g
Distilled water	30.0 ml

Shake for 20 minutes, then add:-

1.0 M NaOH		8.0 ml
Distilled water	to make	50.0 ml

8. JB-4 Plastic embedding medium.

Supplied by Polysciences Limited, Northampton.

a) Catalysed solution A

JB4 solution A	50 ml
JB4 Catalyst powder	0.45 g

Stir for 15 minutes or until clear.

b) Complete resin

Catalysed solution A	42 ml
JB4 solution B	1 ml

9. Toluidine blue staining procedure.

Stain in 1% toluidine blue in 1% sodium tetraborate solution on slide, 5-10 seconds

Rinse in distilled water.

Dry on hotplate.

Mount in DPX.

10. Haematoxylin-phloxine staining procedure.

Stain in Ehrlich's haematoxylin at 40°C	10 min
Rinse in tap water.	
Stain in 1% aqueous phloxine	2 min
Dry on hotplate.	
Mount in DPX.	

APPENDIX II

Preparation of sterile sea water with antibiotic.

Sea water was initially filtered through Whatman No. 1 filter paper, then sterilized by filtration through a Millipore filter of 0.45 um pore size.

Crystamycin (Glaxo Laboratories, Lt., Greenford, Middlesex), a mixture of benzylpenicillin sodium and streptomycin sulphate, was added at the rate of 1 x 800 mg vial per litre of sterile sea water.

APPENDIX III

Reagents used for the demonstration of the uptake of horseradish peroxidase.

1. Sigma Type II horseradish peroxidase was used, at a concentration of 10 mg/ml in sterile sea water.

2. Buffered Hanker-Yates Reagent.

   Hanker-Yates reagent is a mixture of pyrocatechol and para-phenylenediamine (Hanker *et al*, 1977) and was supplied by Polysciences Limited, Northampton.

Tris	1.21 g.
Distilled water	60.0 ml
Adjust to pH 7.6 with 2 M HCl.	
Distilled water	to make 100.0 ml
Hanker-Yates reagent	0.125 g

3. Complete incubation medium.

Buffered Hanker-Yates reagent	100 ml
1% H_2O_2 solution	1 ml

APPENDIX IV

Reagents used for the demonstration of acid phosphatase activity.

1. Stock 0.2 M cacodylate buffer.

Sodium cacodylate trihydrate		4.28 g
Distilled water		60.0 ml
Adjust pH to 7.4 with 2 M HCl.		
Distilled water	to make	100.0 ml

2. Cacodylate-buffered glutaraldehyde.

0.2 M cacodylate buffer, pH 7.4		50 ml
Glutaraldehyde, EM grade, 25%		12 ml
NaCl		3 g
Distilled water	to make	100 ml

3. Cacodylate-buffered osmium tetroxide.

0.2 M cacodylate buffer, pH 7.4		50 ml
Osmium tetroxide crystals		1 g
NaCl		3 g
Distilled water	to make	100 ml

4. Buffer wash solution

0.2 M cacodylate buffer, pH 7.4		50 ml
NaCl		3 g
Distilled water	to make	100 ml

5. Stock 0.2 M Tris/maleate buffer.

Tris	2.4 g
Maleic acid	2.3 g
Distilled water	60.0 ml

Adjust pH to 5.2 with 2 M NaOH.

Distilled water	to make 100.0 ml

6. 0.1 M sodium β-glycerophosphate.

Sodium β-glycerophosphate	3.15 g
Tris/maleate buffer, pH 5.2	to make 100.0 ml

7. 0.02 M lead nitrate.

Lead nitrate	0.66 g
Tris/maleate buffer, pH 5.2	to make 100.0 ml

8. 0.1 M sodium fluoride.

Sodium fluoride	0.42 g
Tris/maleate buffer, pH 5.2	to make 100.0 ml

APPENDIX V

Details of radiochemicals used.

1. Tritiated leucine and glucose were supplied by Amersham International plc., Amersham, Buckinghamshire.

2. Tritiated leucine.

   L-[4,5-^{3}H] leucine, specific activity 130 Curies/mmol was supplied at 1 mCi/ml in sterilized aqueous solution with 2% ethanol added.

3. Tritiated glucose.

   D-[2-^{3}H] glucose, specific activity 10.2 Curies/mmol, was supplied at 1mCi/ml in sterilized aqueous solution.

Key to Lettering of Diagrams and Figures

ab	accumulation body
Ac	amoebocyte
ac	accessory centriole
al	angular inclusion
Al	algal cell
al	annulate lamellae
an	anterior
Ap	process from amoebocyte
as	anterior space
ax	axoneme
ay	atypical yolk granule
Bl	blastocoele
bl	basal lamina
Bm	blastomere
bo	cell boundary
bs	blebs
cb	base of cilium
ce	centriole
cg	compound yolk granule
ch	chromatin, or channel in intercellular junction
ci	cilium
cl	cleft in cytoplasm
co	cortical granule
cp	coated pit, or cytoplasmic packet
Ct	ciliary tract
ct	central point of degenerate area
cu	central unfragmented region
cv	coated vesicle
cy	cytospine
d	desmosome-like intercellular junction
da	dense area
db	dense body
dc	area of dense cytoplasm
dcg	dense-cored granule
de	depression, or dense core
der	dilated ER cisterna
Df	degenerate flagella
DG	degenerate germ cell
dg	dense granule
dm	degenerate mitochondrion
DN	degenerate nucleus
DO	degenerate oocyte
dr	dense region
DS	degenerate sperm
DTe	degenerate testicular cyst
dy	degenerate yolk granule
ea	early compound yolk granule
Ec	ectoderm
En	endoderm
Eo	endodermal portion of oocyte
Ep	'plug' of endodermal cells
ep	epithelial cell process
er	endoplasmic reticulum
ex	extension of mesogloea
f	flagellum
Fb	fish red blood cell
fb	fibrillar material, or nuclear fibrillar body

fe	feltwork of fibrils
fg	fibrillar granule
fi	microfilaments or fibrils
Fl	lower portion of filament
fo	fossa
Fu	upper portion of filament, or furrow
fy	forming yolk granule
g	Golgi complex
ga	granular area
gc	granular core
gl	glycogen
GO	gonad
go	Golgi complex
gr	granule
Gt	cnidoglandular tract
Gv	gastrovascular cavity
hc	heterochromatin
ho	hooped fibrillar material
ib	intercellular bridge
IC	ingested cell
ij	intercellular junctional complex
Im	imperfect mesentery
in	invagination
Is	inner surface of blastula
It	intermediate tract
iy	ingested yolk
iyp	ingested yolk packet
L	lipid droplet
Lc	'light cell'
ld	low-density granule
lf	loosely packed fibrillar granule
li	lipidic inclusion
lm	limiting membrane
LO	large oocyte
lo	lobe
Ls	large lipid sphere
ls	longitudinal section
m	mitochondrion
ma	mitochondrial aggregation
mc	mitochondrial cloud
md	moderate-density granule
Me	mesogloea
Mi	cell undergoing mitosis
Mo	mesogloeal portion of oocyte
mp	muscle process
Ms	mesentery
mt	microtubule
mu	mucus-like material
mv	multivesicular body
N	nucleus
na	nuage
nd	dense form of nuage
ne	nuclear envelope
nf	fibrillar form of nuage
np	particulate form of nuage
nt	nematocyst
nu	nucleolus

Oc	oocyte
ol	oolemma
Oo	possible oogonium
Op	opening in epithellum or mesogloea
op	oocyte process
Ov	ovum or egg
ox	calcium oxalate crystal
pa	small particle
pc	pore complex
pe	peripheral granule
Ph	pharynx
ph	phagosome
pi	coated pit
pl	chloroplast
Pm	perfect mesentery
pn	pyrenoid
po	posterior
pp	periplast
pr	cytoplasmic process
Ps	prospermatogonium
ps	peripheral space, or pseudopodial process
PT	partially emptied testicular cyst
ra	randomly arranged fibrillar material
rb	residual body-like inclusion
rd	rodlet
Re	remnant of sperm
Rm	retractor muscle
Rt	reticular tract
rt	rootlet
s	starch grain
sa	satellite granule
Sc	shrunken cell
sc	synaptinemal complex
se	septae (of desmosome)
sh	membranous sheet
sk	membranous stack
sm	sperm membrane
SO	small oocyte
Sp	spermatogonium
Sr	spirocyst
sr	striated rootlet
ss	starch sheath
St	spermatid
st	stack of Golgi cisternae
sw	swollen region
Sz	spermatozoon
tb	tubule
Te	testicular cyst
th	chromatin thread
Tr	trophonema
tu	cluster of tubules
tv	tubular or vesicular material
un	unstained body

va	vacuole
ve	vesicle
vi	microvilli
vm	vacuole membrane
vr	vesicular region
vs	vesicular granule
wh	whorl
xs	cross or transverse section
y	yolk material in endoderm
yo	yolk
yp	yolk-containing packet

TISSUE & CELL 1984 16 (3) 393–404

ALAN U. LARKMAN

THE FINE STRUCTURE OF MITOCHONDRIA AND THE MITOCHONDRIAL CLOUD DURING OOGENESIS ON THE SEA ANEMONE *ACTINIA*

Key words: Anthozoa, *Actinia fragacea*, mitochondrial cloud, oogenesis, ultrastructure

ABSTRACT. The appearance and arrangement of the mitochondria during all stages of oocyte growth in the sea anemone *Actinia fragacea* (Cnidaria: Anthozoa) have been examined by electron microscopy. In small oocytes, the mitochondria are generally squat, with a dense matrix and numerous cristae, although a proportion may show an unusual arrangement of prismatic cristae. During early oogenesis, the mitochondria tend to be arranged in aggregates rather than randomly scattered, and may be associated with nuage material. With the onset of vitellogenesis, a large mitochondrial aggregate forms next to the nucleus. During early vitellogenesis this aggregate enlarges and comes to resemble the mitochondrial clouds found in some amphibian oocytes. Within the cloud, many mitochondria appear to be highly elongate and irregular in shape. The cloud begins to fragment and disperse midway through vitellogenesis at about the time when cortical granules appear. In fully grown oocytes, some mitochondria may have a much less dense matrix and fewer cristae than the remainder, which may be related to their state of activity.

Introduction

The ova or mature oocytes of many animal species apparently contain very large numbers of mitochondria, which may be sufficient to sustain early embryonic development without significant increase in their number (Billett, 1979). The early germ cells from which the oocytes originate, however, are usually small and contain relatively few mitochondria. Thus the process of oogenesis must often involve a massive accumulation of mitochondria. In some species, intact mitochondria may be supplied from nurse cells which are in cytoplasmic continuity with the growing oocyte, as in the meroistic ovaries of some insects (Mahowald, 1972), and there are isolated reports of mitochondria arising from other oocyte components (Marco and Vallejo, 1976). In many cases, however, it seems likely that the oocyte mitochondria are descended from those of the early germ cells (Billett, 1979), presumably replicating by division. In some species, the replicating oocyte mitochondria spend a period arranged as a large accumulation termed the mitochondrial cloud, and also, perhaps less usefully, known as the Balbiani body or yolk nucleus. Mitochondrial clouds have been most closely studied from the oocytes of anuran amphibians (Billett and Adam, 1976; Callen *et al.*, 1980; Tourte *et al.*, 1981).

Oocytes of the sea anemone *Actinia fragacea* develop without cytoplasmic continuity with other cells. They are closely associated with the somatic epithelial cells of a structure known as the trophonema, but the transfer of large inclusions or intact organelles between trophonema and oocyte has not been observed (Larkman and Carter, 1982). It thus seems probable that the increase in number of the oocyte mitochondria occurs by their replication within the oocyte. This paper describes the ultrastructural features of the oocyte mitochondria and their arrangement throughout oogenesis in this species.

Department of Biological Sciences, Portsmouth Polytechnic, King Henry 1 Street, Portsmouth PO1 2DY, U.K.

Received 24 October 1983.
Revised 1 February 1984.

Actinia fragacea is an intertidal sea anemone which was formerly considered to be a variety of the common beadlet anemone *Actinia equina* (Stephenson, 1935), but which is now thought to be a separate species (Carter and Thorpe, 1981).

Materials and Methods

In order that all stages of oogenesis could be observed, large *Actinia fragacea* individuals were collected at regular intervals over a 2 year period from a restricted area of rocky shore at Wembury, near Plymouth, England. The anemones were dissected and pieces of gonad were removed and fixed in 3% glutaraldehyde in a 0·1 M phosphate buffer containing 3% sodium chloride. The pieces were rinsed, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated using ethanol and embedded in Emix epoxy resin. Sections were cut using glass or diamond knives on an LKB Ultratome III, stained with uranyl acetate and lead citrate, and examined using a Philips EM300 electron microscope.

Results

In *Actinia fragacea*, the sexes appear to be separate and both show an annual cycle of gametogenic activity, although oocyte development, even within a single gonad, may be poorly synchronized. In the population studied, early female germ cells are first noticed in spring, and appear to be spawned in June or July of the following year. Thus the whole process of oogenesis may take a full year, or slightly more for some oocytes. The female germ cells are first apparent as small cells, 5–8 μm in diameter, among the bases of the endodermal epithelial cells of the gonad, and there is evidence that at least some of these very small cells may already be oocytes (Larkman, 1981). Early in their development, at sizes ranging usually from 10 to 30 μm in diameter, the oocytes migrate from the endoderm into the mesoglea of the gonad, usually between June and October. Vitellogenesis may begin prior to entry into the mesoglea, but for most oocytes it only becomes significant after entry (Larkman, 1983). The oocytes grow within the mesoglea throughout the winter and spring, and attain a final size of about 150 μm.

The very smallest oocytes have a high nucleus to cytoplasm ratio, and hence have rather few cytoplasmic organelles. The few mitochondria they contain tend to be dispersed through the cytoplasm (Fig. 1). As the oocytes begin to enlarge, more mitochondria become apparent. They tend to be small and

Abbreviations

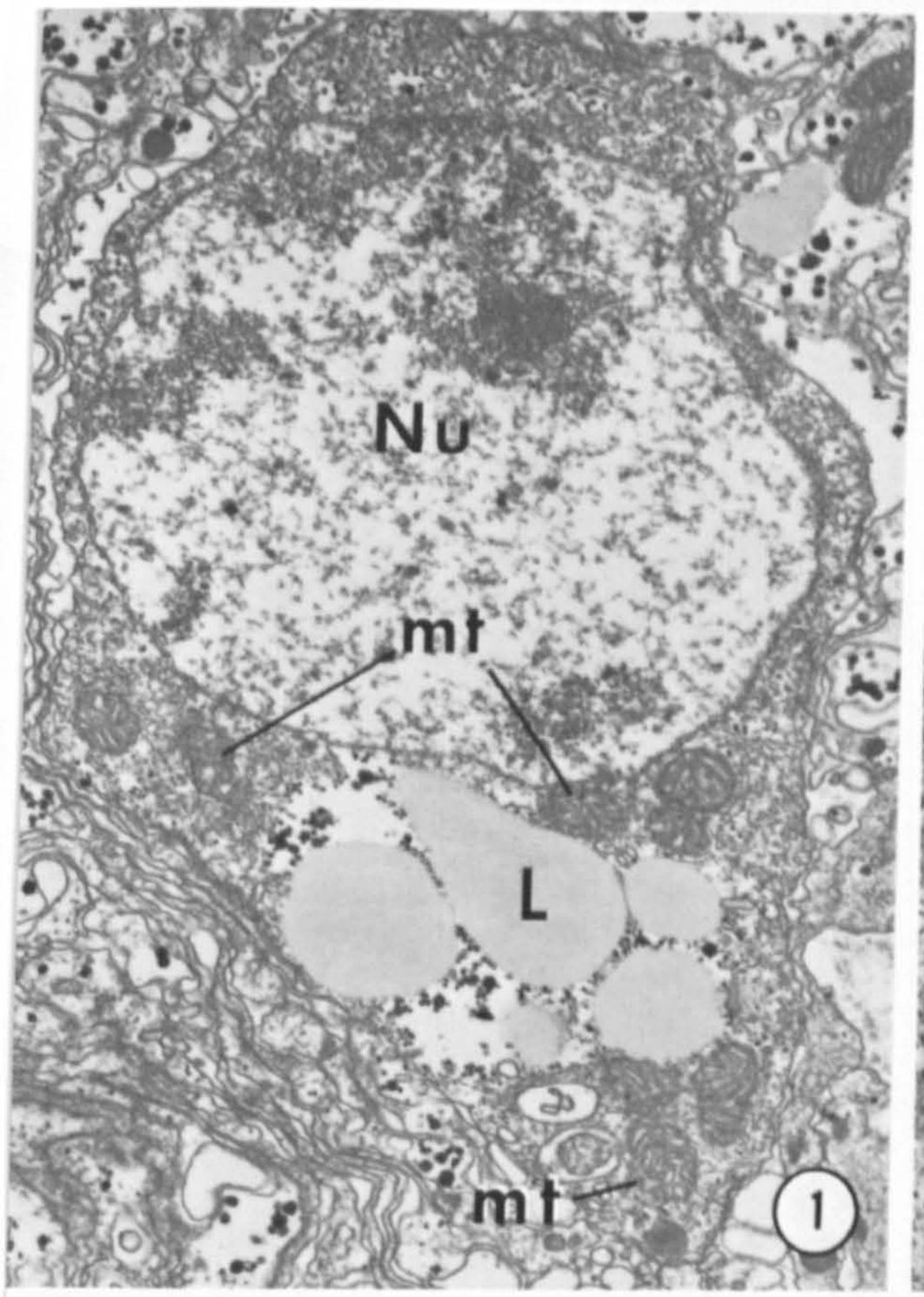
ce	centriole	Me	mesoglea
cm	central matrix area	mt	mitochondria
co	cortical granule	mv	multivesicular body
En	endoderm	na	nuage material
er	endoplasmic reticulum	no	nucleolus
fi	fibrillar granule	Nu	nucleus
gl	glycogen	pc	pore complexes
Go	Golgi complex	yo	yolk granule
L	lipid droplet		

Fig. 1. A very small oocyte, about 6·5 μm diameter. The mitochondria are scattered through the cytoplasm. ×16,000.

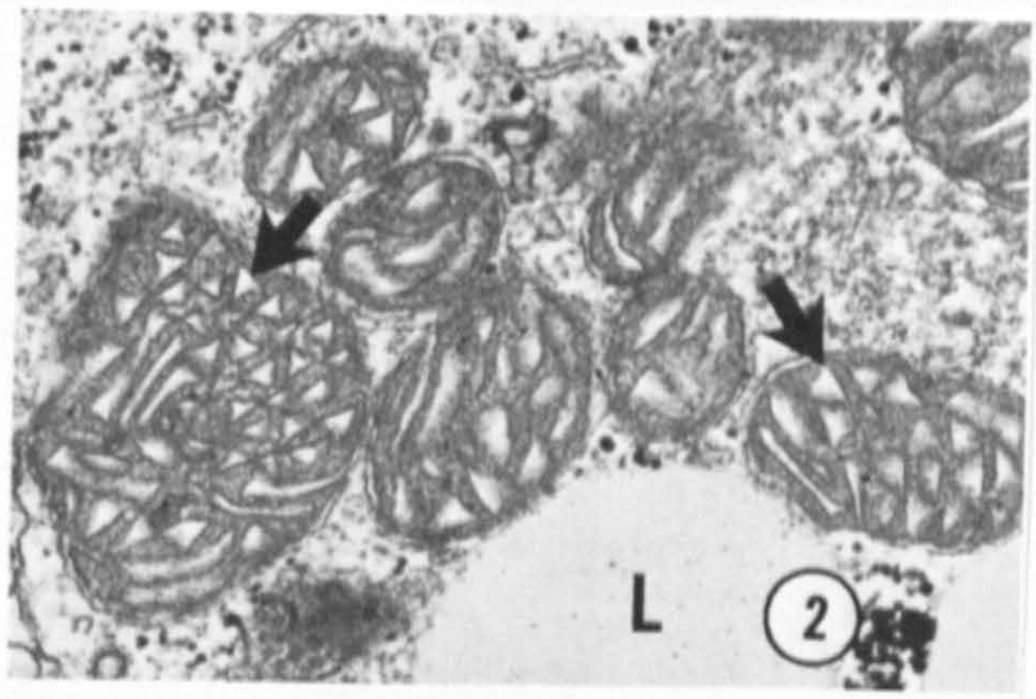
Fig. 2. A group of mitochondria from a small oocyte, showing the arrangement of prismatic cristae (arrowed). ×38,000.

Fig. 3. A group of mitochondria from a small oocyte, one of which contains a central matrix area which is not traversed by the cristae. ×30,000.

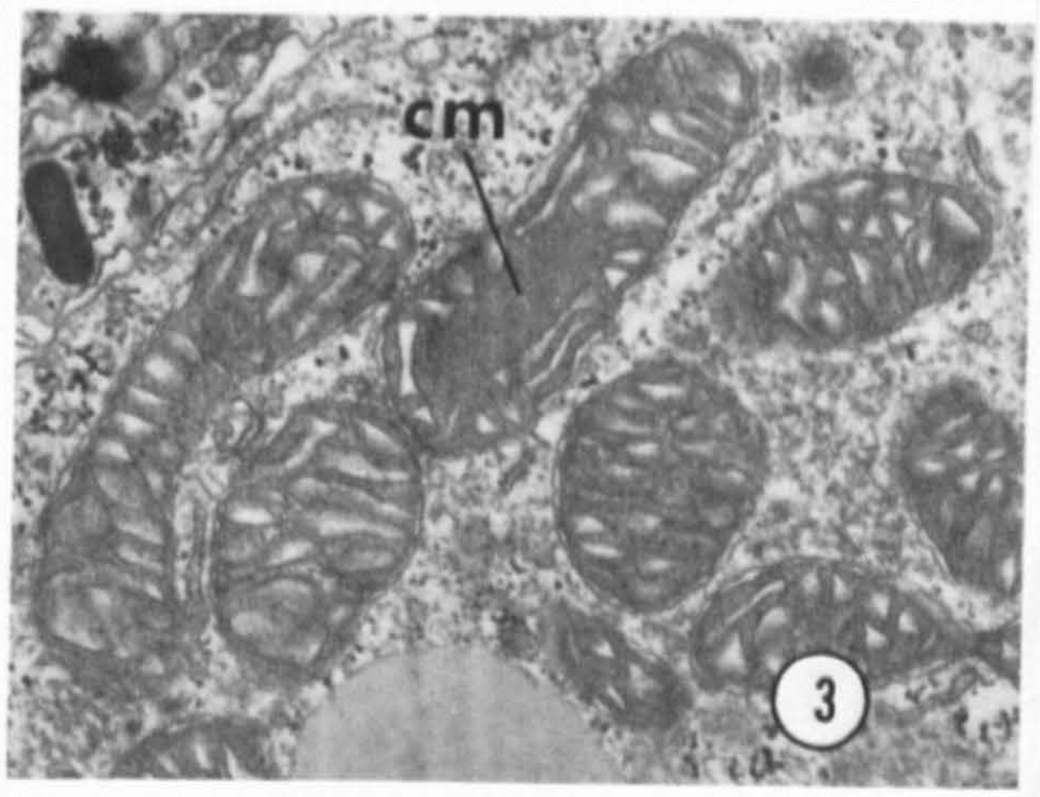
Fig. 4. A 12 μm diameter oocyte in the endoderm, close to the mesoglea. The mitochondrial aggregate lies close to an area of nuage material. ×10,000.



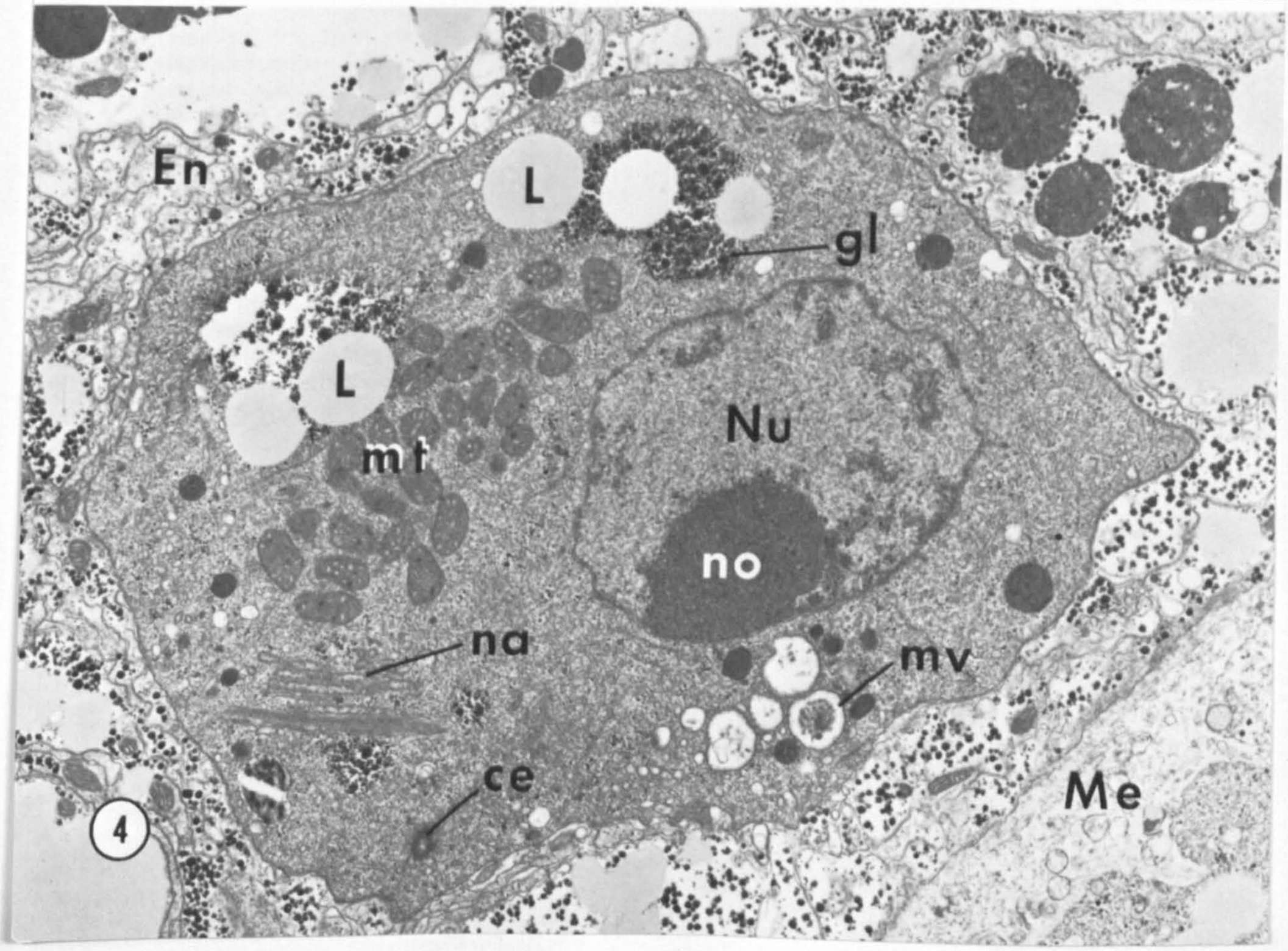
1



2



3



4

rounded, measuring about 0·4×0·6 μm in section. They have a homogeneous dense matrix traversed by numerous narrow cristae. In a proportion of mitochondria, especially in the larger profiles, the cristae show an unusual lattice-like arrangement. Many of these cristae appear triangular in cross-section, and so are thought to be prismatic in three dimensions (Figs. 2, 3). Some mitochondria may also contain a central region of dense matrix material which is not traversed by cristae (Fig. 3). These forms of mitochondria are less common in oocytes larger than about 15 μm in diameter.

As the oocytes grow within the endoderm and the numbers of mitochondria they contain increase, the mitochondria tend to become arranged in groups rather than scattered through the cytoplasm (Fig. 4). These groups are usually found close to the nucleus, but occasionally occur more peripherally in the cytoplasm. These groups do not always contain all the mitochondria of the oocyte; apparently isolated mitochodria are often found.

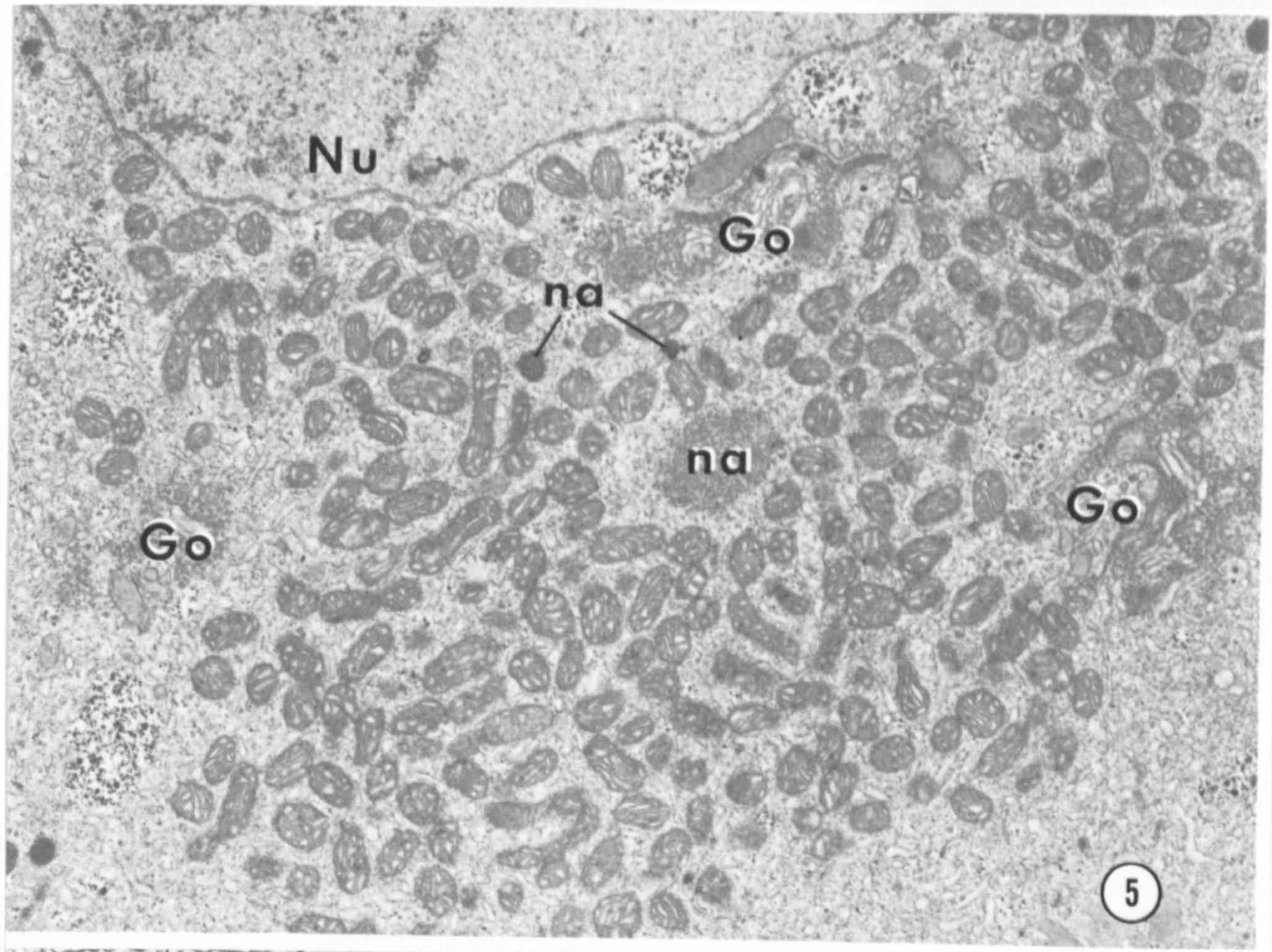
A number of apparently related structures occur in *A. fragacea* oocytes which have also been found in male germ cells but not in other anemone cell types, and which are thought to represent a form of nuage material. The nuage and associated structures have been described in previous publications (Larkman, 1981, 1983) and so will not be considered in detail here. In this paper, all forms of nuage and related structures will simply be referred to as nuage. In small oocytes, nuage is often found associated with groups of mitochondria (Fig. 4). However, actual contact between nuage and mitochondria has not been observed, and nuage is sometimes found apparently unassociated with mitochondria.

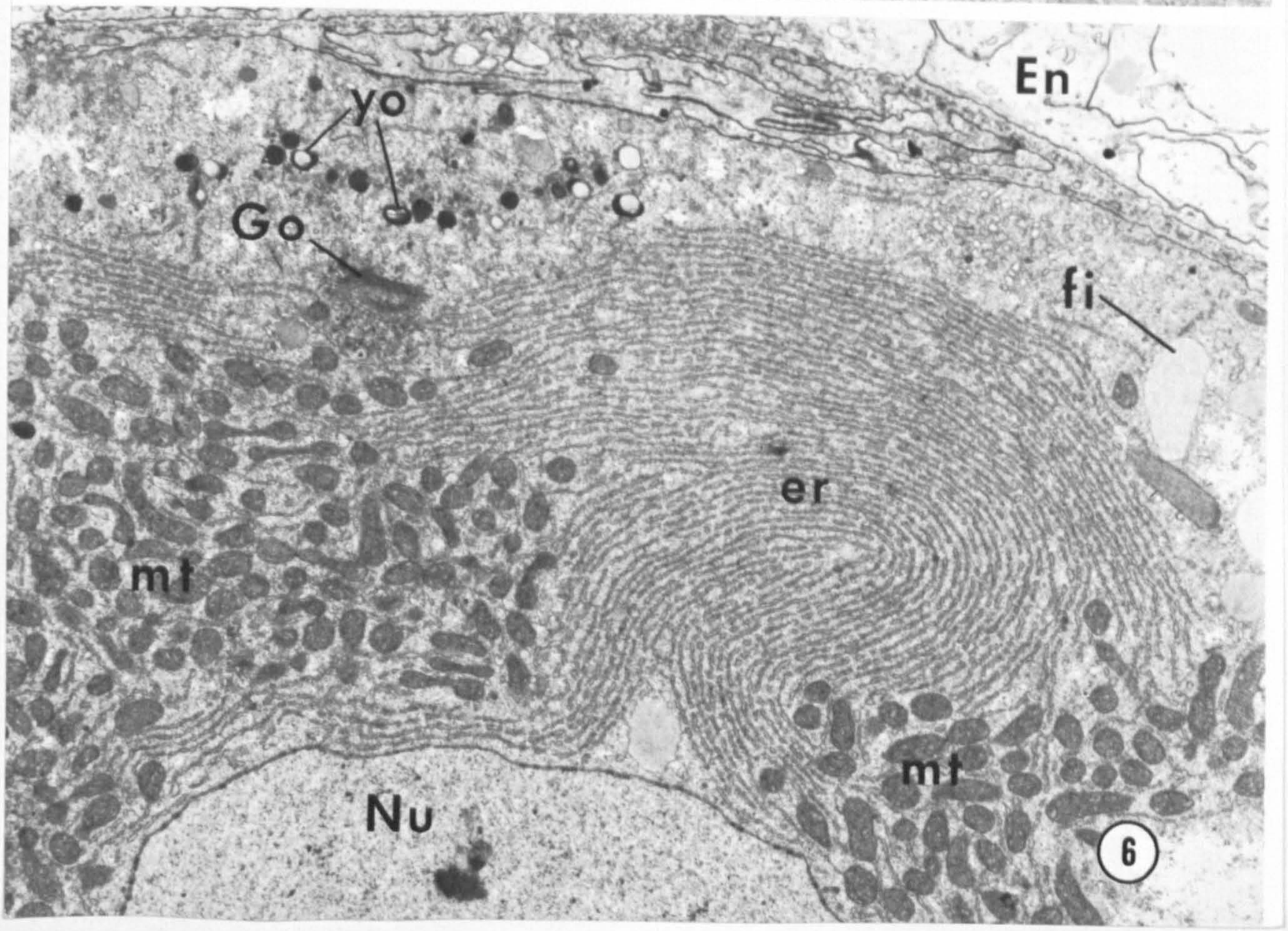
Towards the end of the pre-vitellogenic phase, usually soon after entry into the mesoglea and at an oocyte diameter of 20–30 μm, a group of mitochondria lying close to the nucleus begins to enlarge. This presumably occurs by replication of mitochondria within the group, although other groups and single mitochondria may become incorporated into the group as it expands. The perinuclear group comes to resemble the mitochondrial clouds described for other species (Fig 5). During the early stages of cloud formation, nuage material and sometimes Golgi complexes may be found within it (Fig. 5). As major vitellogenesis gets under way, the cloud enlarges further. At this stage, usually between 30 and 50 μm in diameter, the oocytes develop an extensive band of rough endoplasmic reticulum which encircles a significant part of the nucleus. Occasionally, this band may extend into the perinuclear region occupied by the mitochondrial cloud, and may disrupt it (Fig. 6). More commonly, however, the cloud remains relatively compact, although it may be irregular in outline. The mitochondria are not tightly packed within the cloud, and ribosomes and short lengths of endoplasmic reticulum are found interspersed with them. Later in vitellogenesis, yolk granules and lipid droplets may be found within the cloud (Fig. 7). Nuage and associated structures are rarely found in well-developed clouds in vitellogenic oocytes, however. The cloud reaches its greatest size usually mid-way through vitellogenesis, at an oocyte diameter of perhaps 80 μm, and may itself be 12–15 μm across. While the cloud may contain a high proportion of the mitochondria of the oocyte, small groups and individual mitochondria may still be found elsewhere in the ooplasm.

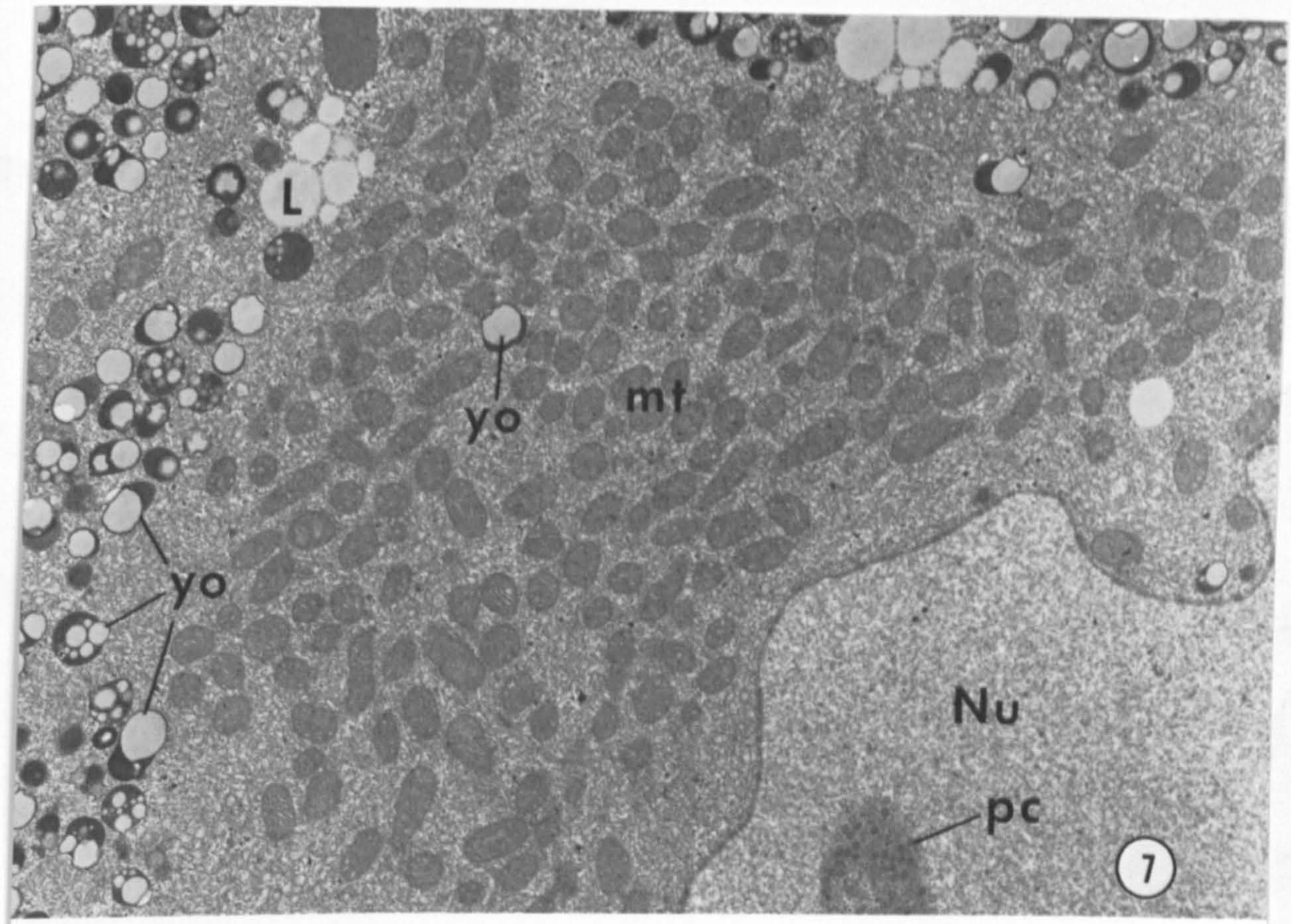
During the early stages of cloud formation, the great majority of the component mitochondria retain their original appear-

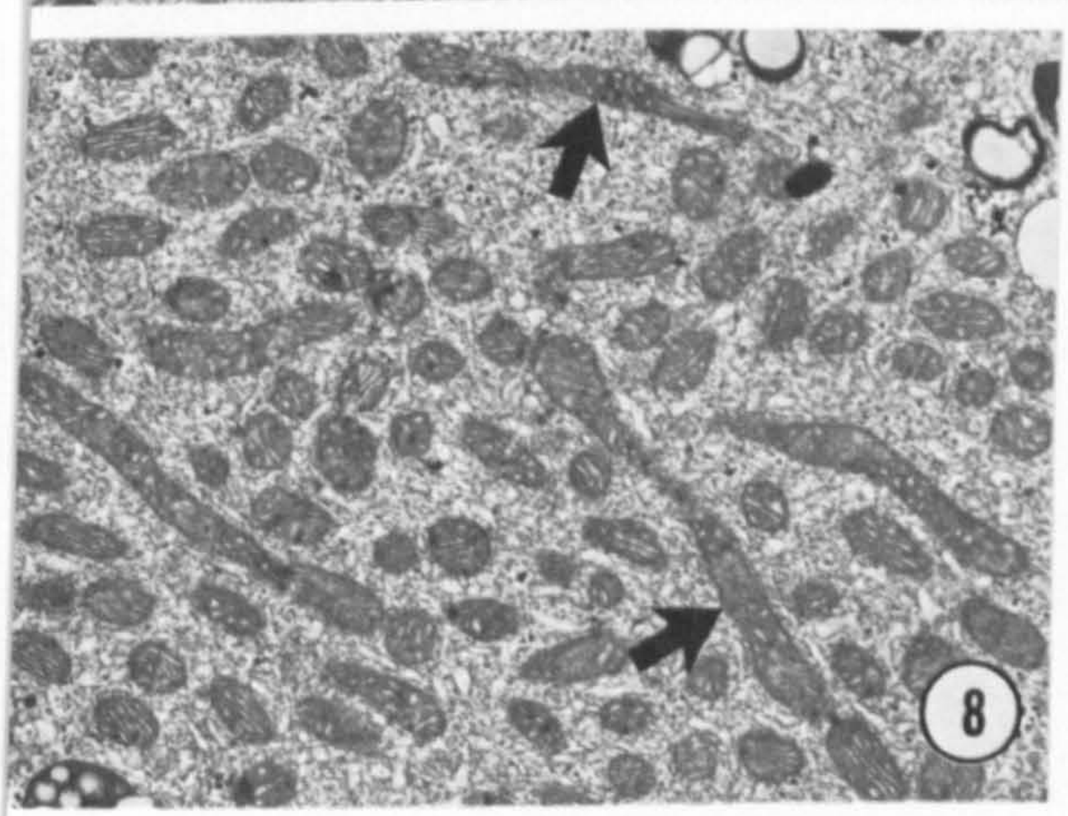
Fig. 5. The mitochondrial cloud next to the nucleus of an oocyte just beginning vitellogenesis. Nuage material is still found within the cloud and Golgi complexes occur around its edge. ×13,000.

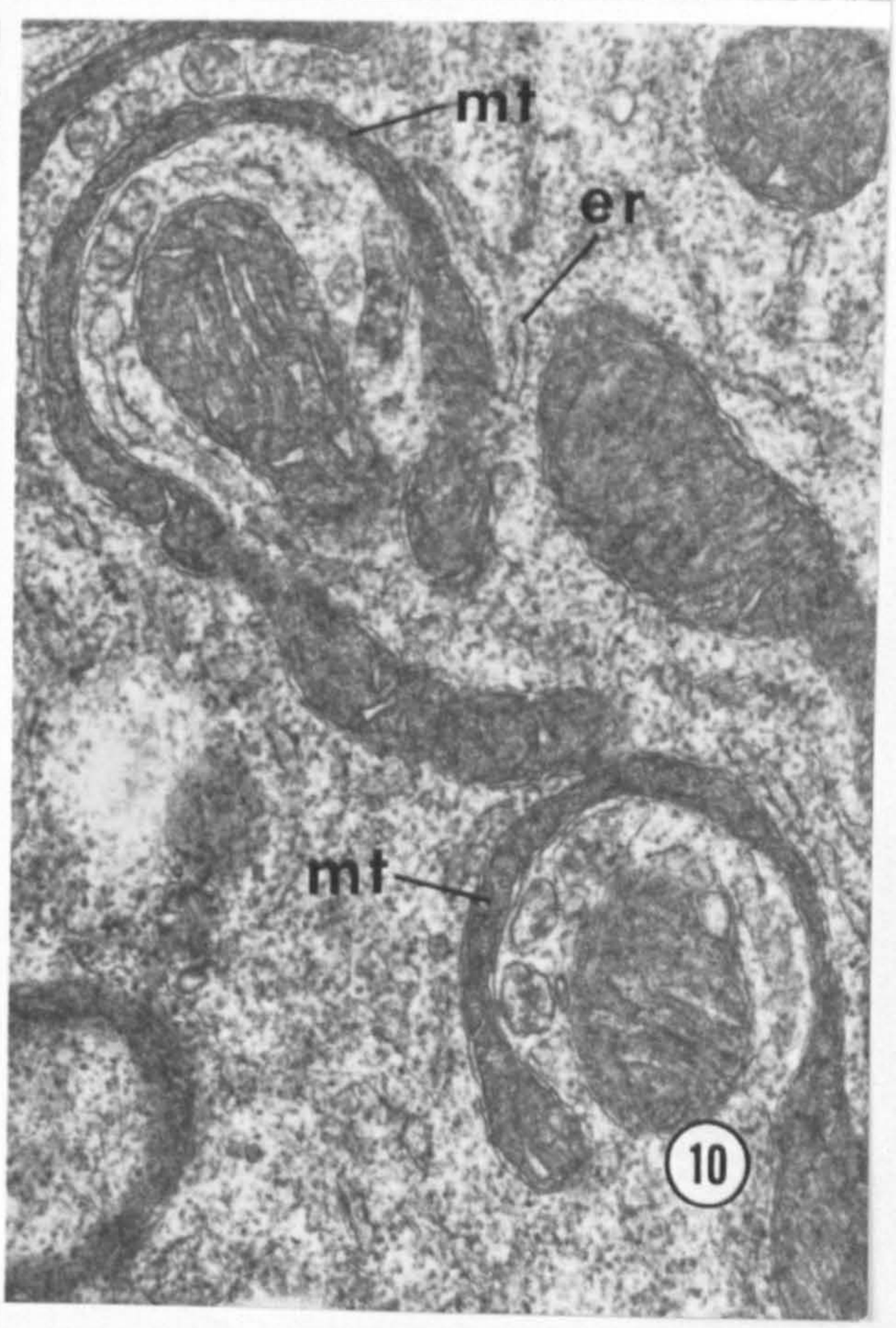
Fig. 6. Part of an early vitellogenic oocyte. A large whorl of perinuclear endoplasmic reticulum has penetrated the mitochondrial cloud. ×7500.

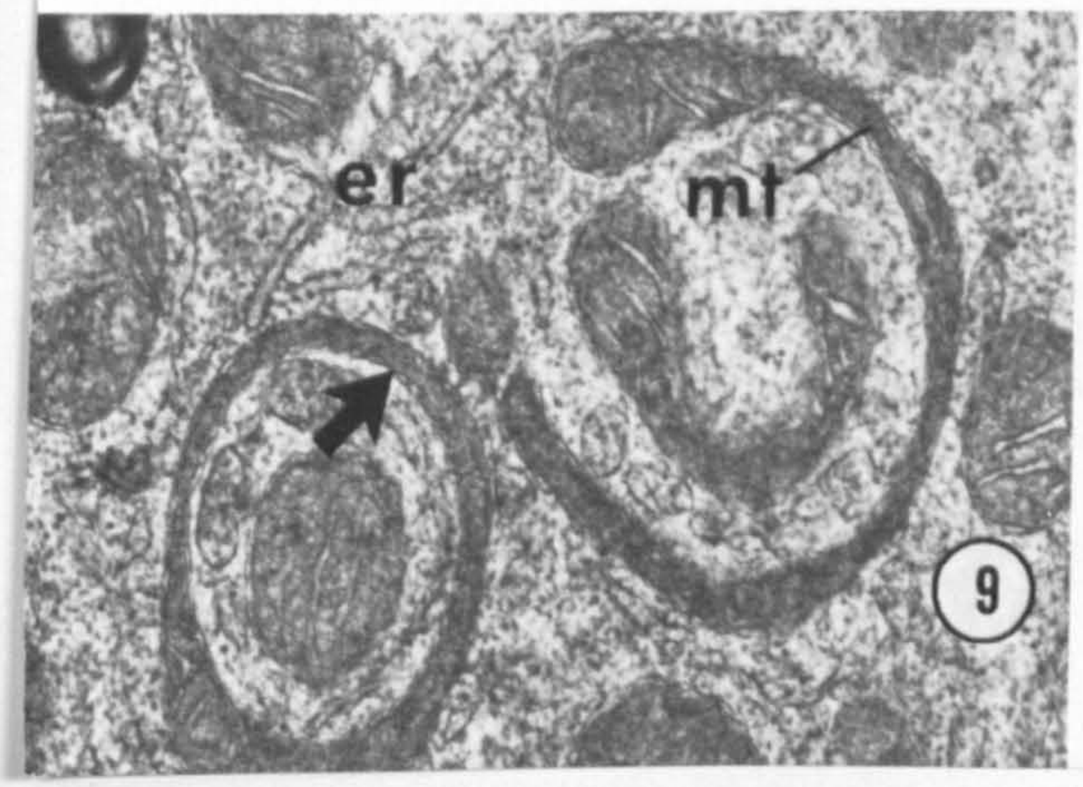

Nu
Go
na
na
Go
Go
5

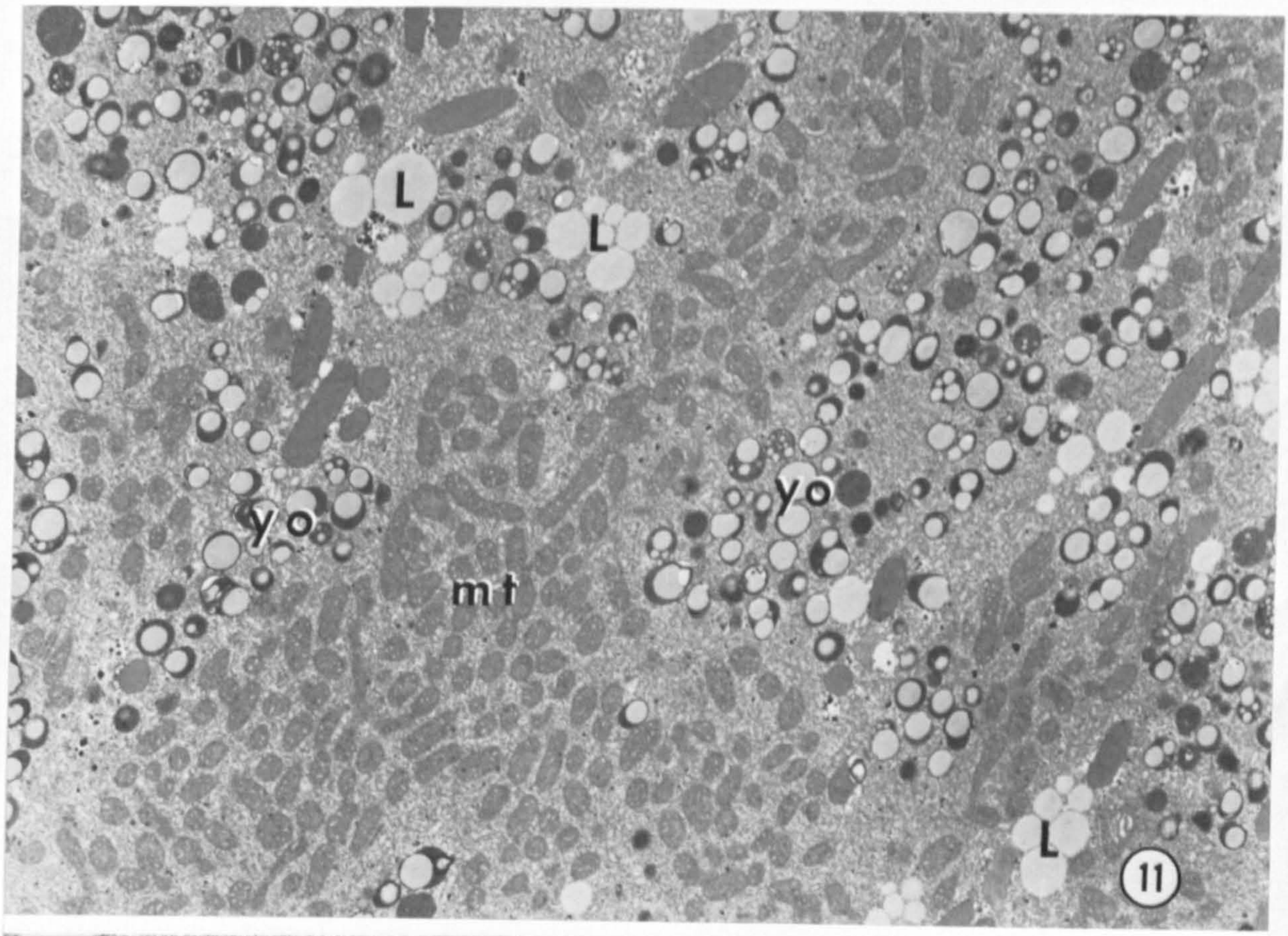

yo
Go
En
fi
er
mt
Nu
mt
6

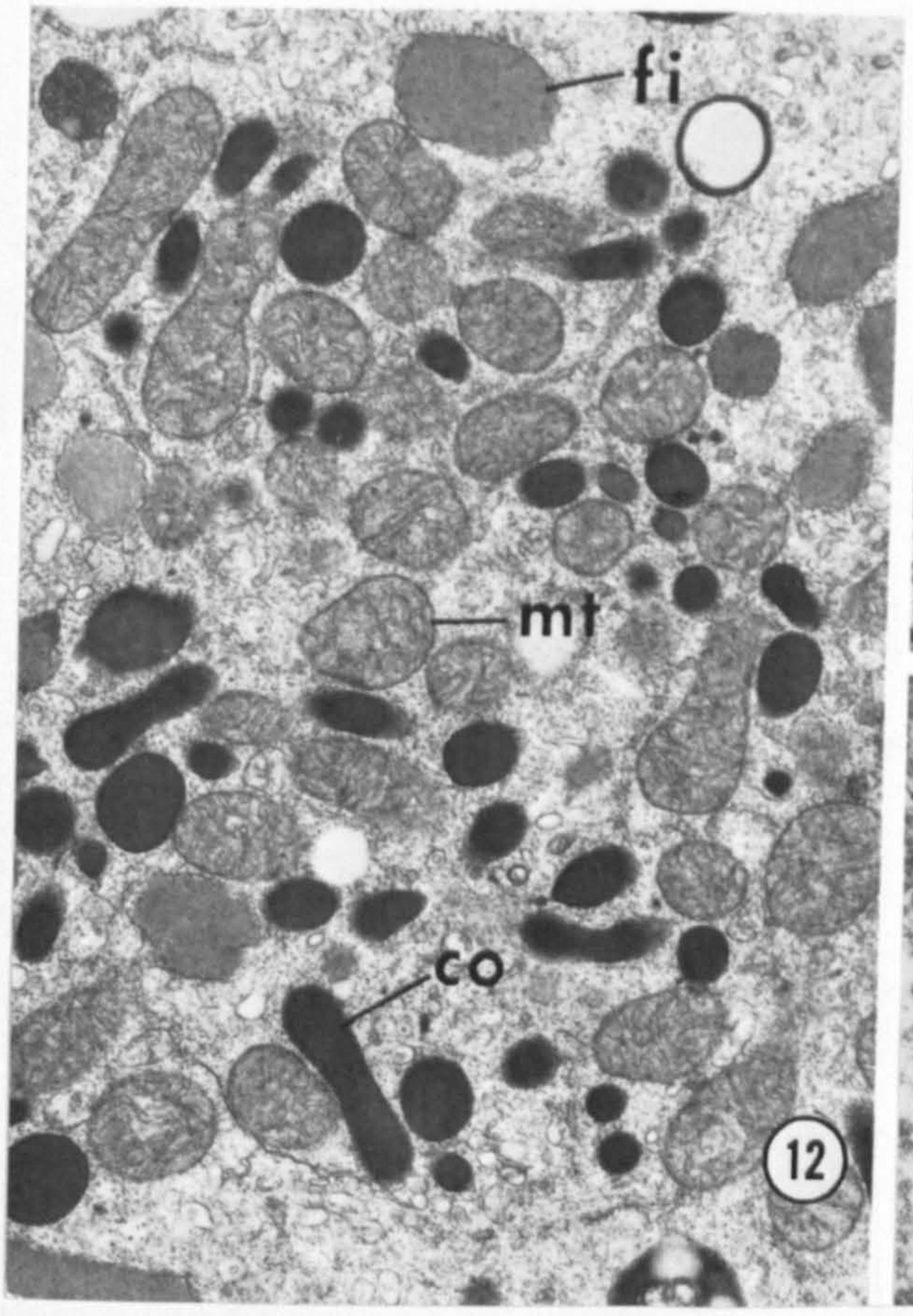

L
yo
mt
yo
Nu
pc
7

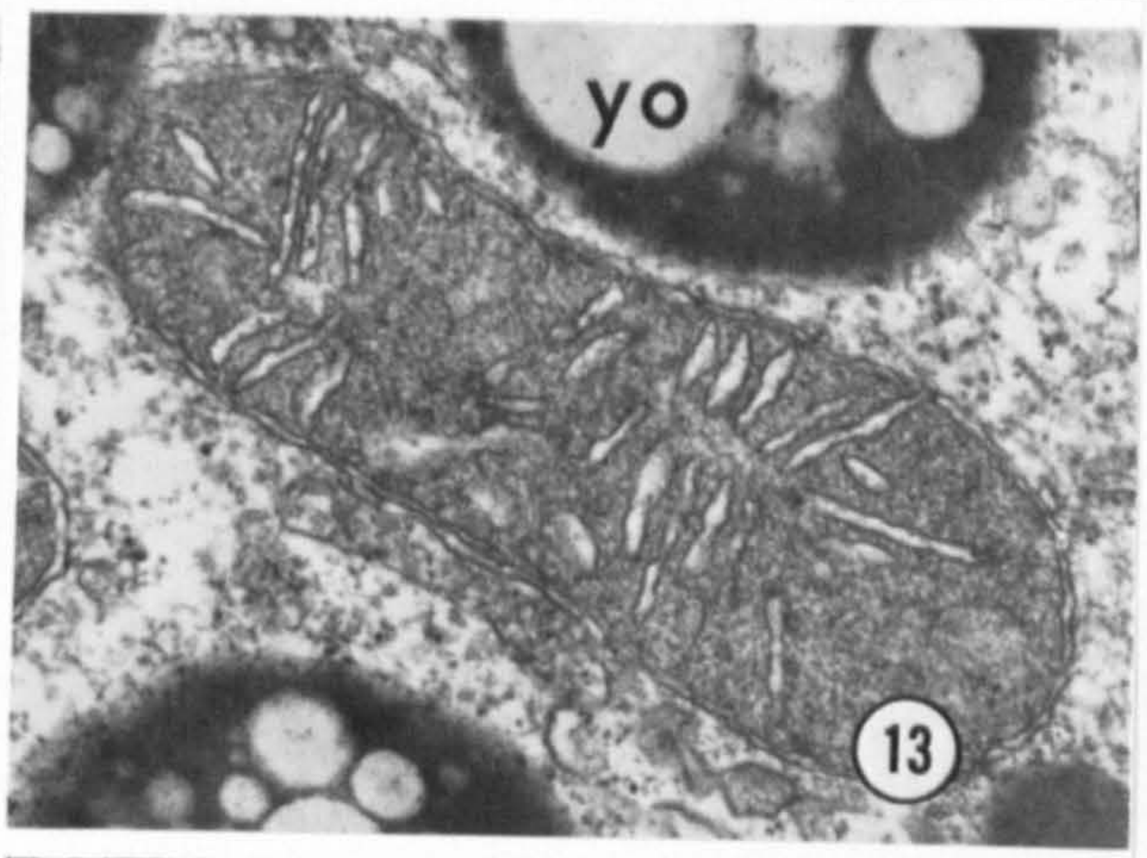

8

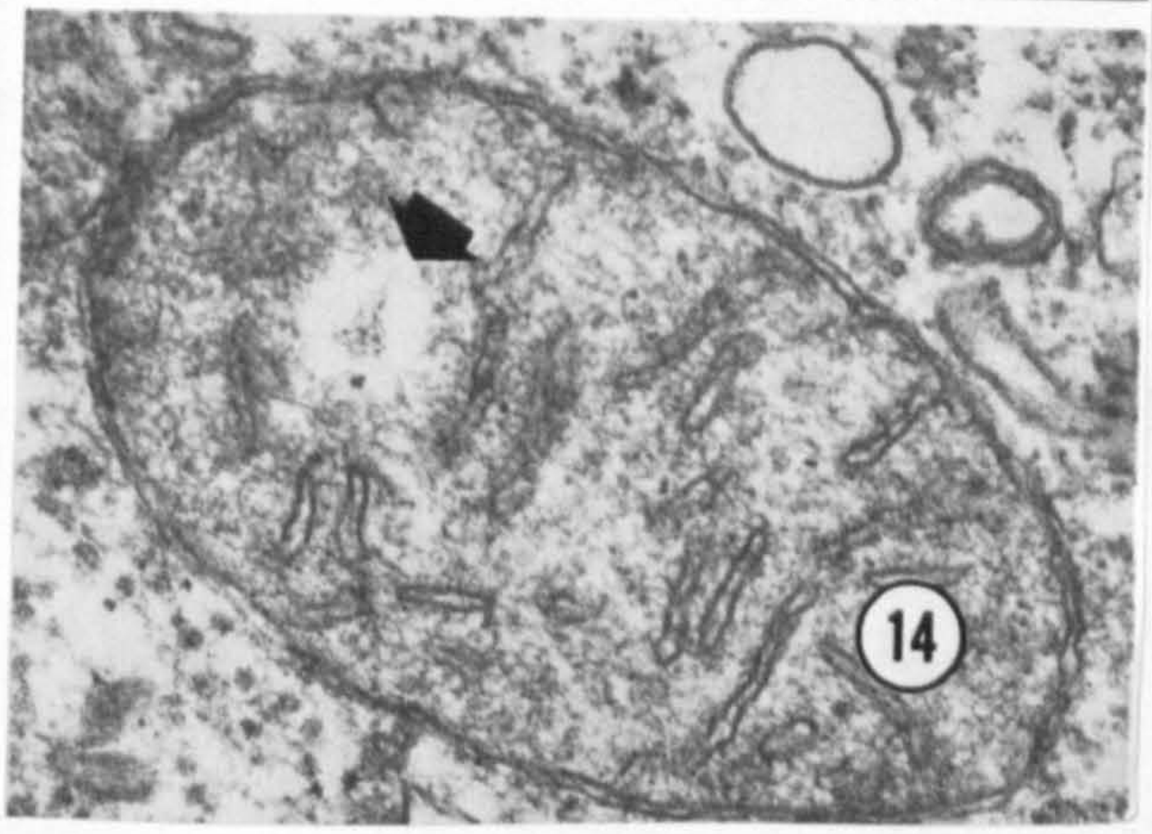

mt
er
mt
10


er
mt
9


L
L
yo
yo
mt
L
11


fi
mt
co
12


yo
13


14

rather different appearance. In these cases, the matrix is very much less electron-dense, and may include areas which appear virtually clear (Fig. 14). The cristae are usually few in number, but are narrow and of fairly constant width. This different appearance of some mitochondria may indicate an alteration in their physiological state, or could merely reflect poor preservation during fixation. However, they may be found in oocytes in which all the other cell components appear to be well preserved.

Discussion

Mitochondria with prismatic cristae, while not widespread, have been reported on a number of occasions, both from vertebrates (e.g. Blinzinger *et al.*, 1965; Morales and Duncan, 1971) and invertebrates (e.g. Fain-Maurel, 1968; Murdock *et al.*, 1977). Some of these mitochondria also contain an array of rodlets, and Murdock *et al.* (1977) have proposed a hypothesis that these rodlets are important in establishing the geometric arrangement of the cristae. The mitochondria with prismatic cristae found in *Actinia fragacea* early oocytes are similar in appearance to those described by Murdock *et al.* from crayfish vas deferens sphincter muscle, but do not appear to possess intramitochondrial rodlets. It therefore seems unlikely that their proposed mechanism is operating in this case. It is interesting that, in both cases, only a proportion of the mitochondria in a given region of tissue exhibit this form; they may be intermingled with mitochondria with a more typical arrangement of cristae.

During the course of oocyte growth in *A. fragacea*, the mitochondria come to form a large mass close to the nucleus, which is similar in appearance to the mitochondrial clouds of other animal species. Mitochondrial clouds have been reported from a number of invertebrate species. Recently Eckelbarger (1979) described the occurrence of a mitochondrial cloud in pre-vitellogenic oocytes of the polychaete annelid *Phragmatopoma lapidosa*. Anderson and Huebner (1968) also note the accumulation of mitochondria at the animal pole of oocytes of the polychaete *Diopatra cuprea*. However, the mitochondrial cloud has been most extensively studied in amphibians, and in particular for the anuran *Xenopus laevis*. In spite of the wide phylogenetic separation, parallels can be drawn and comparisons made between the clouds of *A. fragacea* and *Xenopus*.

In small *A. fragacea* oocytes, mitochondria are often found in clusters which may have a perinuclear location, as is the case in young oocytes of many species (Anderson, 1974). One such perinuclear cluster enlarges and eventually forms the mitochondrial cloud. In *Xenopus*, the cloud arises by the coalescence of several smaller mitochondrial aggregates, which initially are dispersed evenly around the germinal vesicle. They come to form a cap-like structure on one side of the germinal vesicle (Billett and Adam, 1976). In *A. fragacea*, it is not clear whether migration of other mitochondrial groups to join the developing cloud takes place, or whether one perinuclear cluster merely proliferates much more rapidly than the others. In *Xenopus*, during the early stages, the cloud is located in a large depression in one side of the germinal vesicle, which was not observed in *A. fragacea*. Also, Tourte *et al.* (1981) suggest that, in *Xenopus*, there is a precise localization of the cloud with respect to the orientation of the nucleus and the Golgi apparatus during early oogenesis. No favoured position for the cloud on the surface of the nucleus could be discerned during the present study.

The mitochondrial cloud in *A. fragacea* appears to be less regular in shape and less clearly defined than in *Xenopus*. There, the cloud is roughly spherical, can be distinguished with the light microscope within the intact oocyte, and can be removed relatively intact by microdissection (Billett and Adam, 1976). In *A. fragacea*, the cloud, especially late in its development, may be ragged in outline, and bands of yolk granules or endoplasmic reticulum may extend into it.

In *Xenopus*, the cloud, in addition to mitochondria, may contain large numbers of small vesicles, 0·1–0·2 μm in diameter. The relative number of these vesicles increases as the cloud enlarges (Billett and Adam, 1976). No such vesicles were observed within the cloud in *A. fragacea*, although it may contain ribosomes and short, randomly arranged cisternae of rough endopasmic reticulum.

Aggregates of mitochondria are found associated with nuage material in *Xenopus*

primordial germ cells and oogonia (Al-Mukhtar and Webb, 1971), but this associaion is not a characteristic feature of the mitochondrial cloud in the oocyte (Billett and Adam, 1976). An association between mitochondria and nuage occurs in *A. fragacea* but seems less intimate than in *Xenopus*, since direct contact between the two was never observed, and areas of nuage were found apparently not in association with mitochondria. A point of similarity is that the association is more noticeable during the early stages of oogenesis. Nuage may be found among mitochondrial groups in small oocytes and in clouds early in their development, but is not normally seen in large mitochondrial clouds. Billett and Adam (1976) conclude that it is more reasonable to suppose that the nuage material may be some kind of germ cell determinant rather than a substance essential for mitochondrial replication. In *A. fragacea*, nuage material identical to that seen in oocytes is also found in early male germ cells (Larkman, 1981, 1983), which do not undergo massive mitochondrial proliferation. This finding might also argue for a wider role for nuage than involvement in replication of mitochondria.

In *Xenopus*, the cloud is thought to be the site of rapid mitochondrial proliferation, and is an active site of mitochondrial DNA synthesis (Billett, 1979; Tourte *et al.*, 1981), although the level of activity of the cloud may vary considerably with the age of the female frog (Callen *et al.*, 1980). Billett and Adam (1976), using a combination of conventional transmission electron microscopy, high voltage electron microscopy of thick sections, and scanning electron microscopy of isolated clouds, concluded that the *Xenopus* cloud consisted of a tangled mass of very long mitochondria, reminiscent of spaghetti, rather than individual short mitochondria. Although only conventional TEM of thin sections was employed in the present study, the available evidence points to there being a possibly similar situation in the mitochondrial cloud of *A. fragacea*. Highly elongate mitochondria, and curved or irregular mitochondria profiles, were frequently encountered in well-developed clouds, but were rarely observed prior to cloud formation or after its dispersal. The upper limit of the lengths of these elongate mitochondria could not be estimated, but, given their usually tortuous shape, is likely to be in excess of the lengths seen in profile in thin sections. Circular or ring-shaped mitochondrial profiles, as found in *A. fragacea*, have not been described from *Xenopus* clouds. They have, however, been observed in pre-vitellogenic oocytes of the mollusc *Ilyanassa obsoleta* (Taylor and Anderson, 1969), and Anderson (1974) indicates that 'donut-shaped' mitochondria may occur during oocyte development in many animals.

The timing of the development of the mitochondrial cloud is rather different in *A. fragacea* and *Xenopus*. In *A. fragacea* the cloud may become apparent at about the time of the onset of vitellogenesis, and may persist as a recognizable, although more diffuse, structure well into vitellogenesis, until at least the time of cortical granule appearance. However, the sequence of events in *A. fragacea* oogenesis appears to be subject to some variation. In *Xenopus*, the cloud forms, grows and disperses all within the pre-vitellogenic phase of oocyte growth. The mechanism of yolk formation is very different in the two species, which may make different metabolic demands upon the oocyte. In *Xenopus*, yolk formation is thought to be heterosynthetic, with yolk protein synthesis taking place remote from the oocyte, in the liver (Wallace and Dumont, 1968), and there is a well-defined pre-vitellogenic phase. In *A. fragacea*, the growing oocyte is well endowed with synthetic organelles such as rough endoplasmic reticulum and Golgi complexes, and it is likely that significant amounts of yolk protein synthesis take place within the oocyte itself (Larkman, 1980). The pre-vitellogenic phase is brief and poorly defined, some oocytes beginning to accumulate yolk granues at a small size, sometimes prior to entry into the mesoglea (Larkman, 1983). Subsequently, the oocytes grow slowly, and may be active in vitellogenesis for an extended period, perhaps as long as nine months.

The finding that fully grown *A. fragacea* oocytes may contain a proportion of mitochondria of unusual appearance may simply be the result of variable fixation and so have no functional significance. However, since other oocyte components appear well preserved, their different appearance may reflect a different physiological state of some mitochondria. It has been suggested that oocyte mitochondria serve two purposes,

firstly, that of providing for the metabolic needs of the oocyte and, secondly, to serve as a store of mitochondria to meet the early requirements of the developing embryo (Billett, 1979). Thus oocytes may contain a mixture of mitochondria, possibly differing both in structure and function. The metabolic needs of a fully grown oocyte may be very different from those of a growing vitellogenic oocyte, especially one engaged in autosynthesis of yolk material such as *A. fragacea*. It may be that when the demands of yolk production decrease, some oocyte mitochondria enter a state of lower activity, which may be reflected morphologically.

In the present study, no evidence was obtained that mitochondria enter the *A. fragacea* oocyte from outside, or are produced from other cytoplasmic structures. Mitochondrial proliferation within the oocyte appears to result in the formation of a large aggregate of possibly elongate mitochondria which may be termed a mitochondrial cloud, a structure not previously reported from coelenterate oocytes. Interestingly, the mitochondrial cloud of this lower metazoan shows many similarities with the clouds described for oocytes of some amphibian species.

References

Al-Mukhtar, K. A. K. and Webb, A. C. 1971. An ultrastructural study of primordial germ cells, oogonia and early oocytes in *Xenopus laevis*. *J. Embryol. exp. Morph.*, **26**, 195–217.

Anderson, E. 1974. Comparative aspects of the ultrstructure of the female gamete. *Int. Rev. Cytol.*, Suppl. **4**, 1–70.

Anderson, E. and Huebner, E. 1968. Development of the oocyte and its accessory cells of the polychaete *Diopatra cuprea* (Bosc). *J. Morph.*, **126**, 163–197.

Billett, F. S. 1979. Oocyte mitochondria. In *Maternal Effects in Development* (eds D. R. Newth and M. Balls), pp. 147–166. Cambridge University Press, Cambridge.

Billett, F. S. and Adam, E. 1976. The structure of the mitochondrial cloud of *Xenopus laevis* oocytes. *J. Embryol. exp. Morph.*, **33**, 697–710.

Blinzinger, K., Rewcastle, N. B. and Hager, H. 1965. Observations on prismatic-type mitochondria within astrocytes of the Syrian hamster brain. *J. Cell Biol.*, **25**, 293–303.

Callen, J. C., Dennnebouy, N. and Mounolou, J. C. 1980. Development of the mitochondrial mass and accumulation of mtDNA in pre-vitellogenic stages of *Xenopus laevis* oocytes. *J. Cell Sci.*, **41**, 307–320.

Carter, M. A. and Thorpe, J. P. 1981. Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific. *J. mar. biol. Ass. U.K.*, **61**, 79–93.

Eckelbarger, K. J. 1979. Ultrastructural evidence for both autosynthetic and heterosynthetic yolk formation in the oocytes of an annelid (*Phragmatopoma lapidosa*: Polychaeta). *Tissue & Cell*, **11**, 425–443.

Fain-Maurel, M. A. 1968. Variabilité de la structure mitochondriale dans les mucocytes des glandes salivaires de *Limnea stagnalis* L. (Gastropode Pulmoné). *C. r. hebd. Séance. Acad. Sci.*, **267**, 1614–1616.

Larkman, A. U. 1980. Ultrastructural aspects of gametogenesis in *Actinia equina* L. In *Developmental and Cellular Biology of Coelenterates* (eds P. Tardent and R. Tardent), pp. 61–66. Elsevier/North-Holland Biomedical Press, Amsterdam.

Larkman, A. U. 1981. An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria: Anthozoa). *Int. J. Invertebr. Reprod.*, **4**, 147–167.

Larkman, A. U. 1983. An ultrastructural study of oocyte growth within the endoderm and entry into the mesoglea in *Actinia fragacea* (Cnidaria: Anthozoa). *J. Morph.*, **178**, 155–177.

Larkman, A. U. and Carter, M. A. 1982. Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a nutritive function. *Int. J. Invertebr. Reprod.*, **4**, 375–379.

Mahowald, A. P. 1972. Oogenesis. In *Developmental Systems: Insects* (eds S. J. Counce and C. H. Waddington), pp. 1–47. Academic Press, New York.

Marco, R. and Vallejo, C. G. 1976. Mitochondrial biogenesis during *Artemia salina* development: storage of precursors in yolk platelets. *J. Cell Biol.*, **70**, 321a.

Morales, R. and Duncan, D. 1971. Prismatic and other unusual arrays of mitochondrial cristae in astrocytes of cats and hamsters. *Anat. Rec.*, **171**, 545–558.

Murdock, L. L., Cahill, M. A. and Reith, A. 1977. Morphometry and ultrastructure of prismatic cristae in mitochondria of a crayfish muscle. A hypothesis of the structural principle. *J. Cell Biol.*, **74**, 326–332.

Stephenson, T. A. 1935. *The British Sea Anemones*, Vol. 11, 426 pp. Ray Society, London.

Taylor, G. T. and Anderson, E. 1969. Cytochemical and fine structural analysis of oogenesis in the gastropod, *Ilyanassa obsoleta*. *J. Morph.*, **219**, 211–247.

Tourte, M., Mignotte, F. and Mounolou, J. C. 1981. Organization and replication activity of the mitochondrial mass of oogonia and pre-vitellogenic oocytes in *Xenopus laevis*. *Devl. Growth Differ.*, **23**, 9–21.

Wallace, R. A. and Dumont, J. N. 1968. The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*. *J. Cell Physiol.* (Suppl. 1), **72**, 73–89.

TISSUE & CELL 1984 16(1) 125–130

A. U. LARKMAN and M. A. CARTER

THE APPARENT ABSENCE OF A CORTICAL REACTION AFTER FERTILIZATION IN A SEA ANEMONE

Key words: *Actinia fragacea*, cortical granules, cytospines, fertilization, ultrastructure.

ABSTRACT. Eggs, embryos and larvae of the intertidal sea anemone *Actinia fragacea* were obtained from spontaneous spawnings in the laboratory and have been examined by scanning and transmission electron microscopy. The eggs average 150 μm in diameter and are covered by tufts of large microvilli known as cytospines, but are not surrounded by a jelly layer or a vitelline coat. The cortical layer of the egg contains large numbers of dense, homogeneous cortical granules. The surface layers of cleavage and blastula stage embryos are similar in composition to those of unfertilized eggs in that the cytospine tufts remain intact and the number of cortical granules remains apparently undiminished. No major discharge of cortical granules indicative of a cortical reaction can have occurred. During gastrulation, many embryos take up large numbers of sperm by a process resembling phagocytosis. These sperm undergo breakdown in the superficial regions of the embryos. The cortical granules persist well into larval life, and their function is unknown.

Introduction

The eggs of sea anemones contain cortical granules (Dewel and Clark, 1974; Schäfer and Schmidt, 1980; Larkman, 1980), as do those of many other animals, including some more advanced invertebrates and many vertebrates including amphibians and mammals (Guraya, 1982). In many species, these granules are discharged to the exterior of the egg soon after fertilization as part of a cortical reaction which helps to prevent the entry of further sperm, which might have a deleterious effect on the embryo (Schuel, 1978; Shapiro and Eddy, 1980). An ultrastructural study of early development in the intertidal sea anemone *Actinia fragacea* was undertaken in order to investigate cortical granule activity in this lower metazoan.

Materials and Methods

Male and female *A. fragacea* spawned spontaneously in closed aquarium tanks in the laboratory on several occasions during the summer of 1982. Series of samples of the resulting eggs, embryos and larvae were fixed and processed for transmission electron microscopy according to the procedure described in a previous paper (Larkman, 1981) except that, after osmication, the specimens were pelleted in agar so that large numbers of each stage could be handled conveniently. Material was processed for scanning electron microscopy by fixation and dehydration as for TEM, followed by drying by the critical point method and sputter-coating with gold/palladium. Specimens were observed using a JEOL JEM 35 scanning electron microscope.

Those embryos which were allowed to remain in the aquarium tanks developed apparently normally and formed free-swimming planula larvae with very little mortality (estimated at less than 10%). Many of these planulae went on to settle and metamorphose into juvenile anemones.

Department of Biological Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth PO1 2DY, U.K.

Received 16 September 1983.

Results

The eggs of *A. fragacea* are isolecithal and

average approximately 150 μm in diameter. There is no vitelline coat or other extracellular investment visible around the spawned egg. The surface of the egg is, however, covered with tufts of large microvilli known as cytospines (Figs. 1, 2). These extend 10 μm or more from the surface of the egg, and may afford it a degree of protection from abrasion and mechanical damage. The cortical region of the egg, to a depth of some 5 μm, is virtually devoid of yolk granules, but contains large numbers of cortical granules. These are dense, homogeneous, membrane-bound bodies which tend to be slightly elongate, averaging 0·4×0·7 μm in size. They are not arranged as a single row, but form a layer five to eight granules deep, interspersed with smaller numbers of fibrillar granules. Granules of much lower electron density are occasionally found close to the egg membrane. These may represent stages in the discharge of the cortical granules or possibly the fibrillar granules to the outside of the egg. This discharge appears to occur at a very low frequency even prior to spawning in oocytes still within the gonad, as has previously been reported for another species of anemone by Dewel and Clark (1974).

The events of fertilization have not yet been observed in detail, but examination of subsequent stages has yielded important information about the process. The surface of cleavage stage embryos (Fig. 3) is almost unchanged in appearance from that of the recently spawned egg. Tufts of cytospines still cover the surface of the blastomeres and, significantly, the cortical granules are still present in apparently undiminished numbers. Discharging granules are still occasionally seen, but at very low frequency, similar to that of the freshly spawned egg or developing oocyte. No massive discharge of cortical granules indicative of a cortical reaction can have taken place at fertilization.

Cleavage proceeds and a hollow blastula is produced with little apparent change in the composition of the surface layers of the embryo, and with no obvious decrease in the number of cortical granules. However, at gastrulation, within about 12 hr of spawning, a remarkable process occurs. Spermatozoa are taken up by many of the gastrulae over their whole surface, until their cortical layers may contain large numbers of sperm (Figs. 4, 5). The process of sperm uptake resembles phagocytosis. Sperm heads are enveloped by pseudopodial processes from the surfaces of the gastrula cells (Fig. 4) and are drawn into the embryo. This process does not involve membrane fusion between sperm and embryo, and the sperm become enclosed in individual membrane-bound vacuoles. The long sperm tails may protrude for some time before they also are engulfed, and tails are often seen tightly coiled within the vacuoles. The sperm-containing vacuoles do not appear to enter beyond the peripheral regions of the embryo.

During gastrulation, the embryo develops cilia and then becomes a swimming larva.

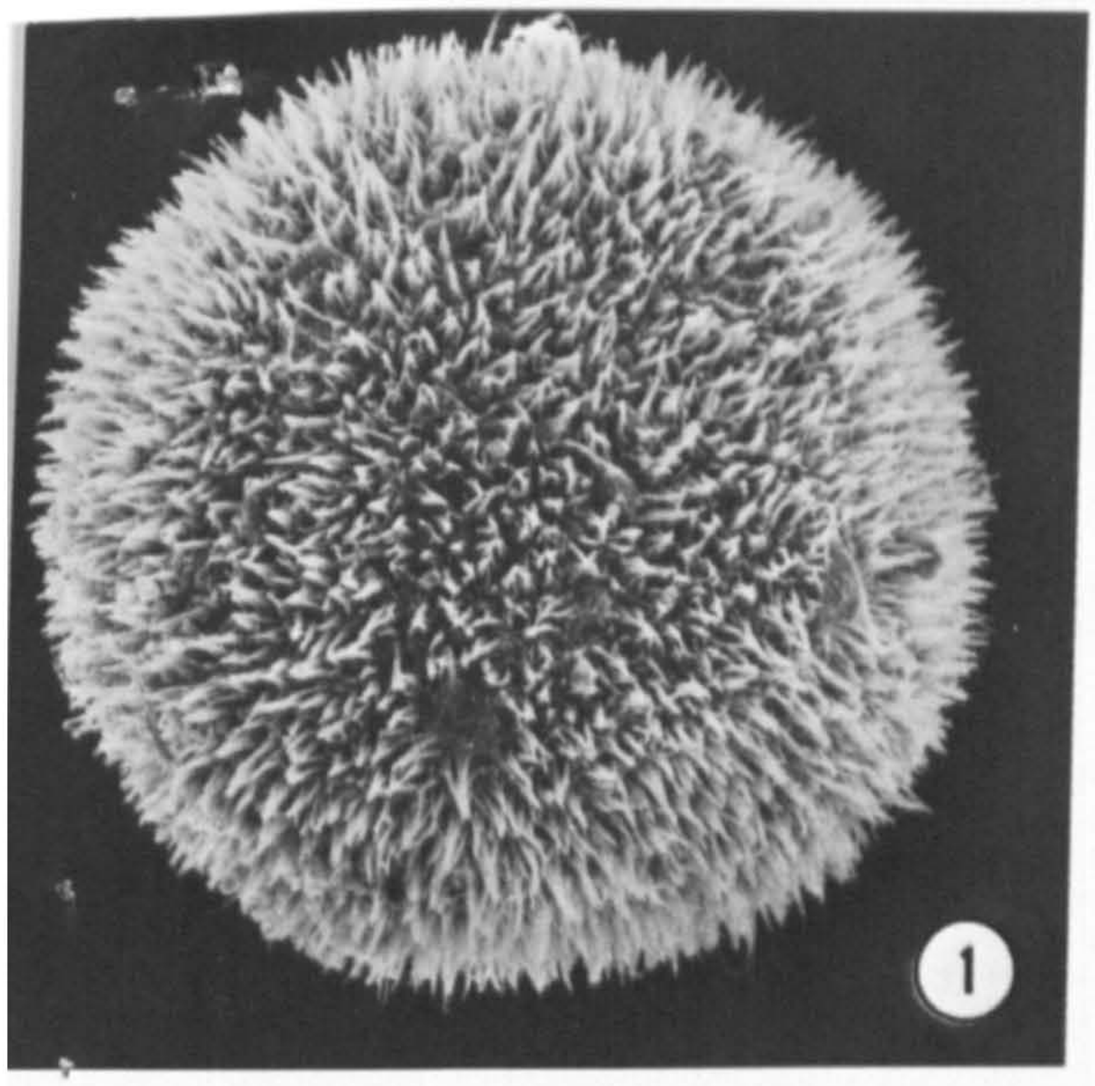
Fig. 1. SEM of a recently spawned egg, showing the tufts of cytospines covering its surface, and the absence of a jelly layer or vitelline coat. ×900.

Fig. 2. TEM of the periphery of an uncleaved egg, through a tuft of cytospines (cy). The cortical ooplasm contains numerous dense cortical granules (cg) and a smaller number of fibrillar granules (fg). ×9000.

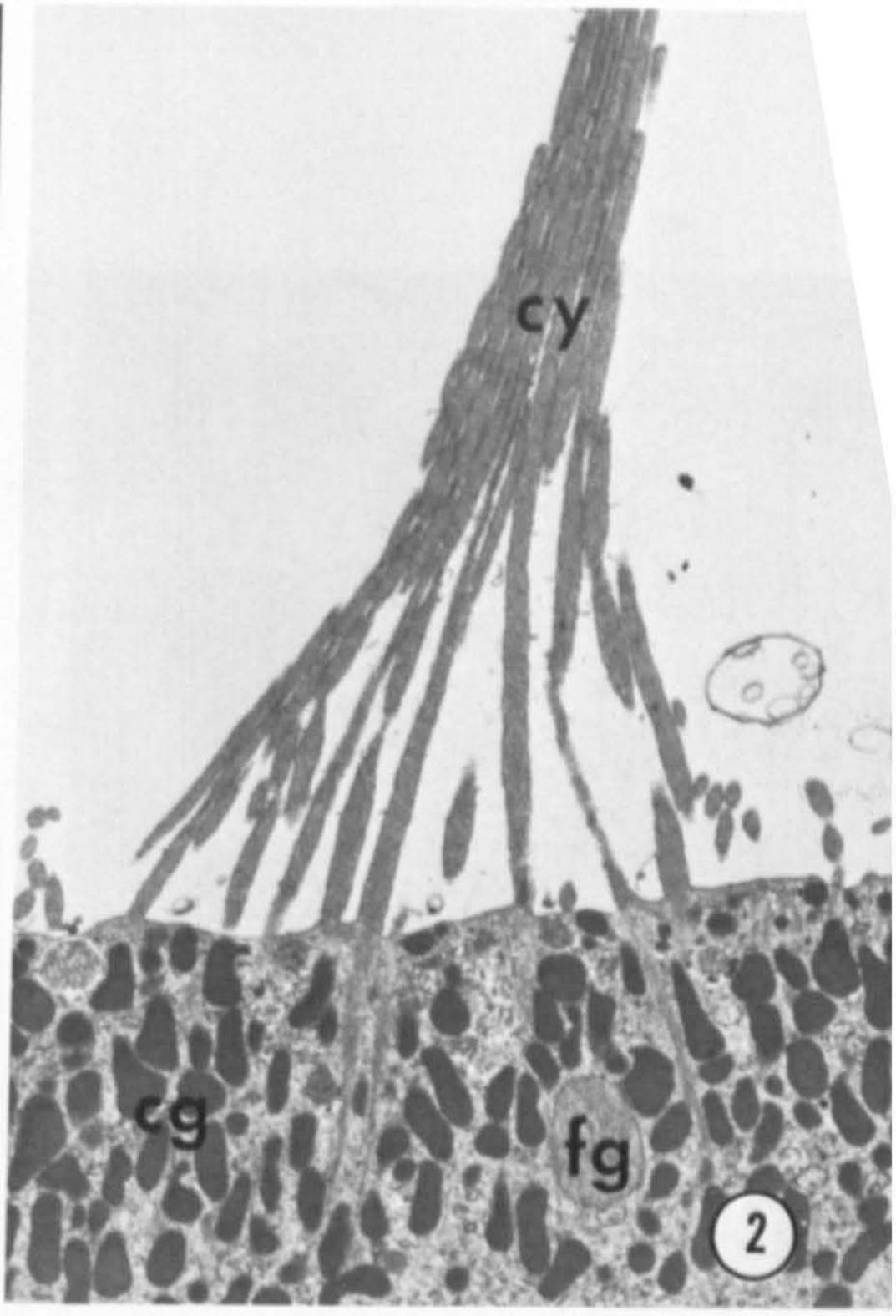
Fig. 3. The edge of a cleavage stage embryo. The two cleavage blastomeres (Cb) still contain cortical granules (cg), and the furrow between them contains cytospines (cy). ×9500.

Fig. 4. The surface of a gastrula cell in the process of engulfing a sperm (sp). A pseudopodial process (ps) has surrounded the sperm head prior to ingestion. The gastrula cells still bear cytospines (cy). ×30,000

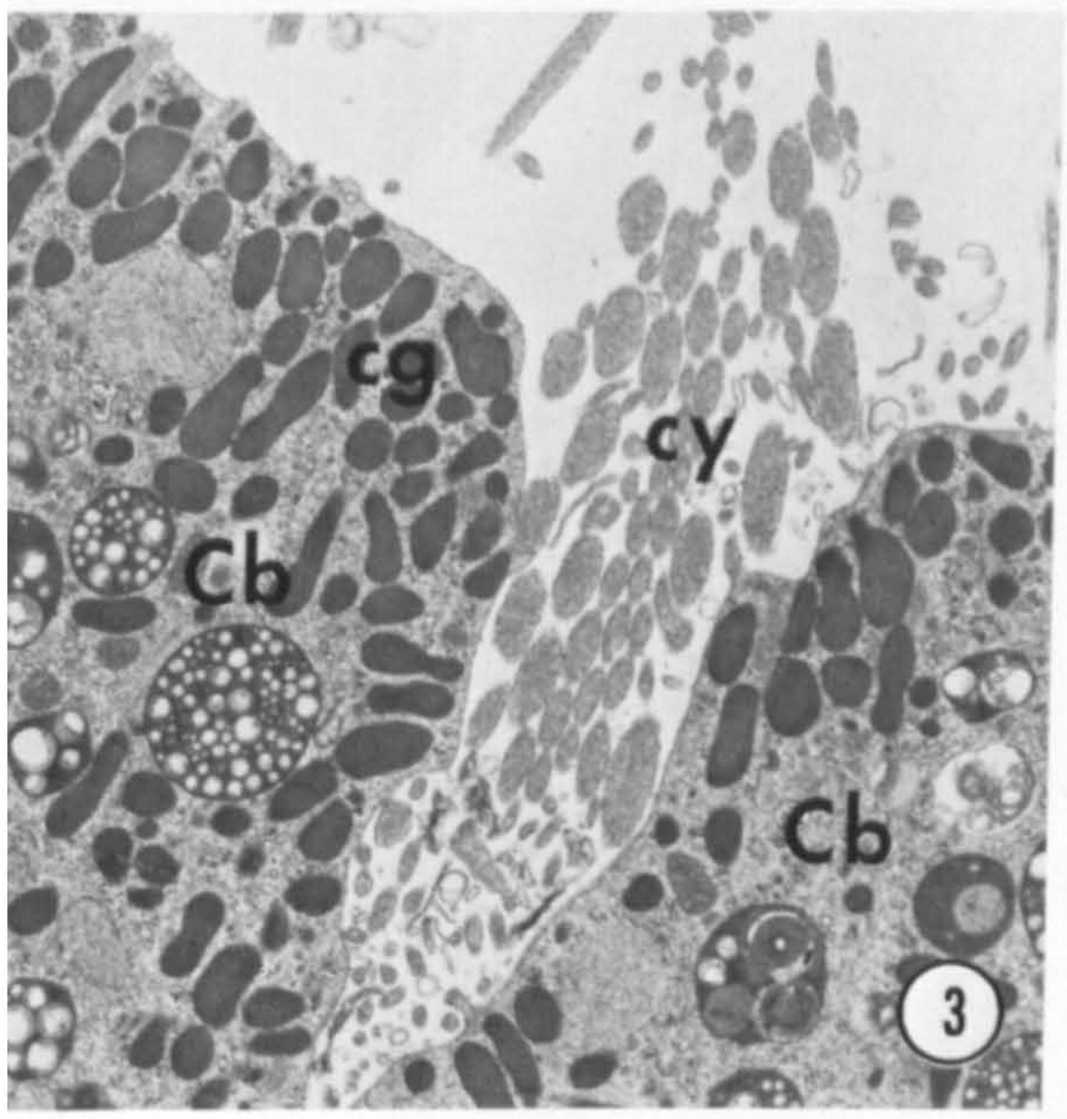
Fig. 5. The periphery of a gastrula stage embryo showing cytospines (cy), cortical granules (cg) and several ingested sperm (sp) near the surface. ×11,000.



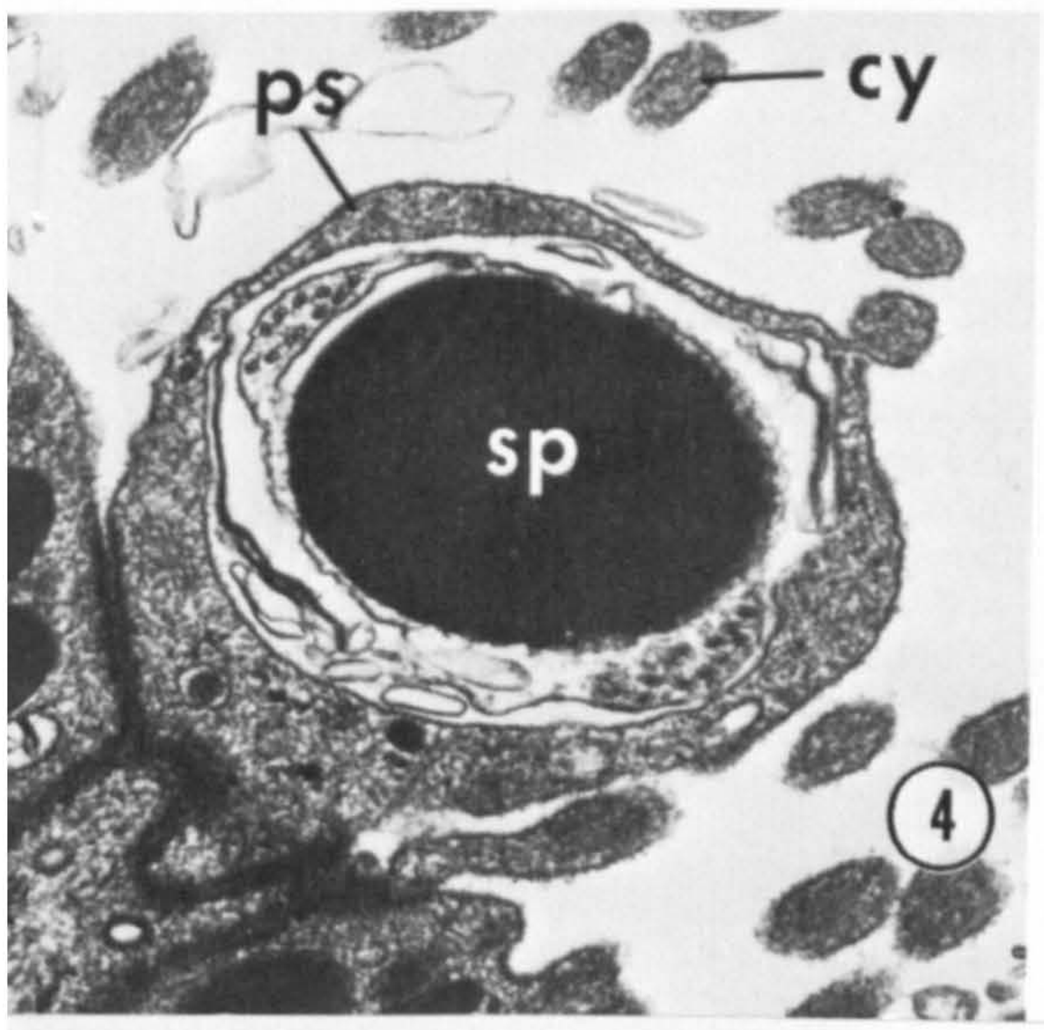
1



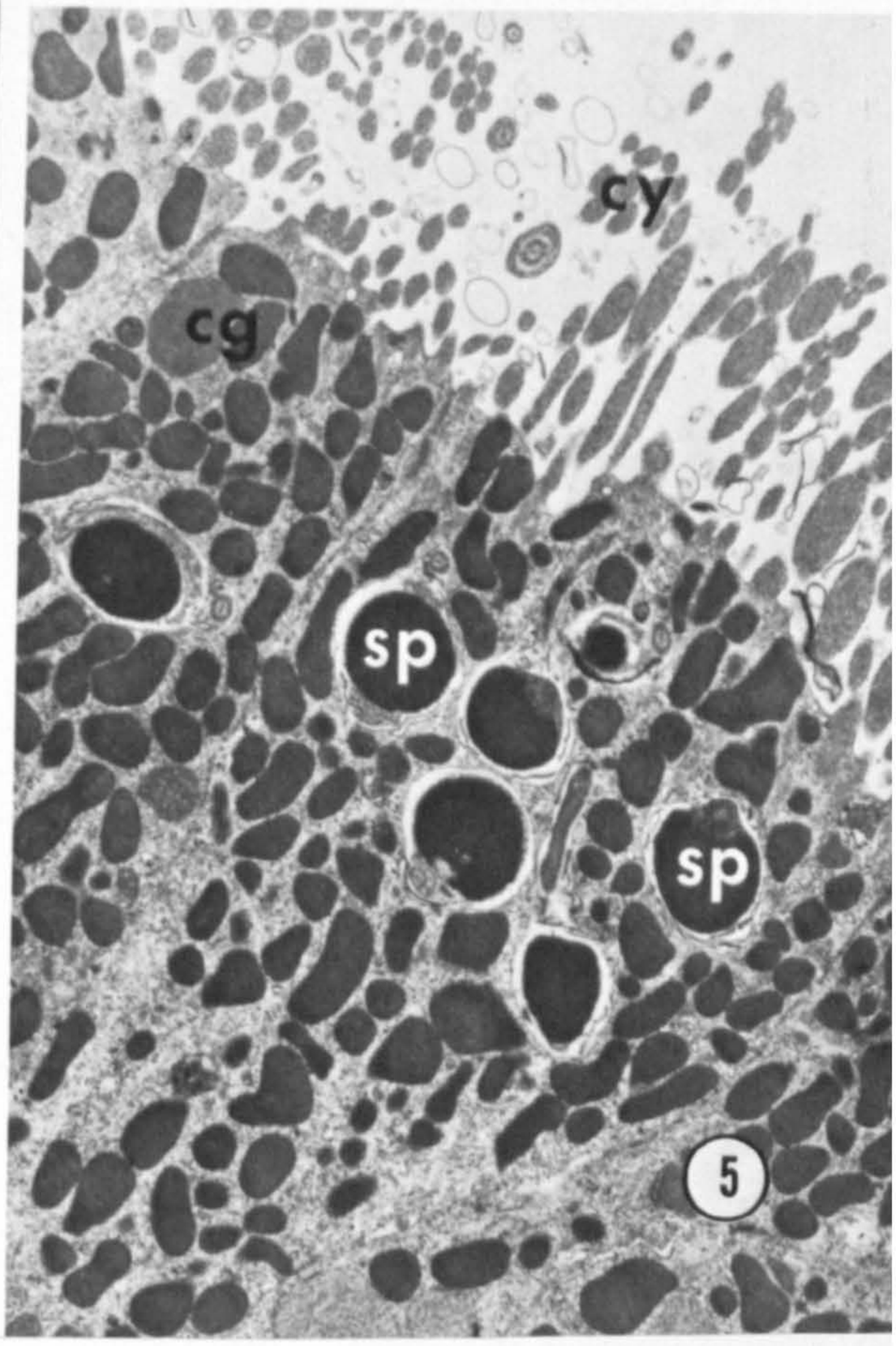
2



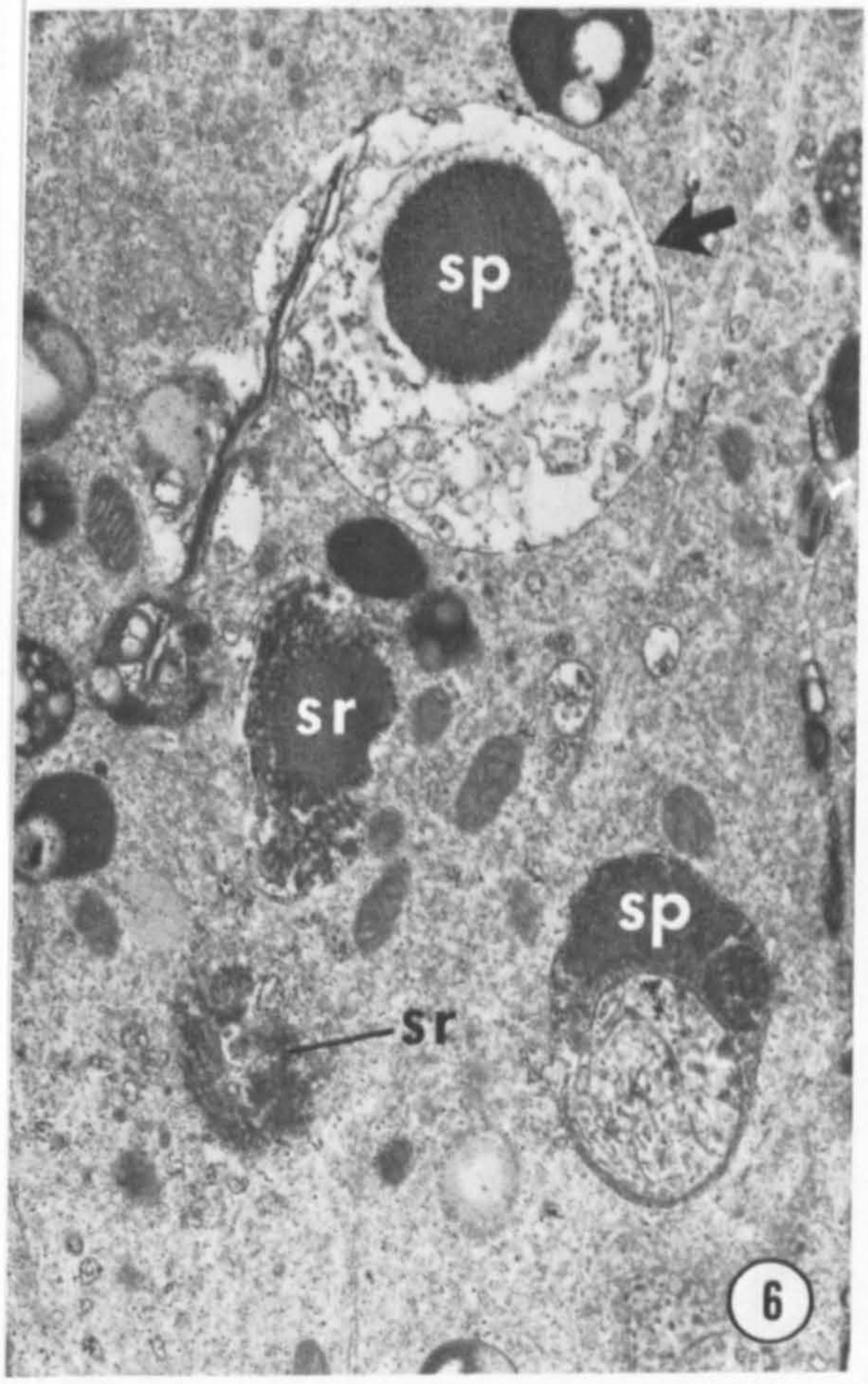
3



4



5



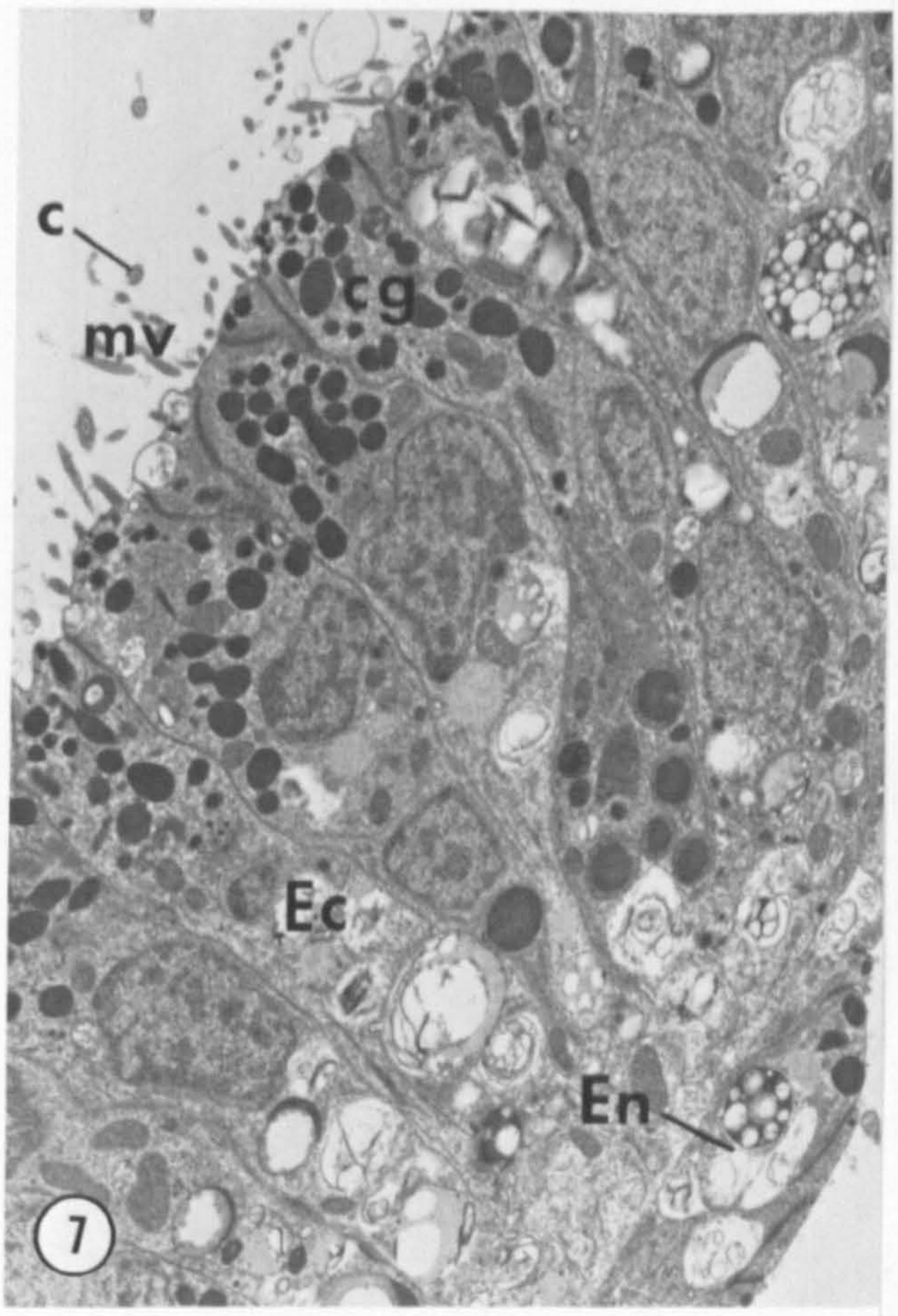


Fig. 6. Stages in the breakdown of ingested sperm. Initially the sperm (sp) are enclosed in a membrane-bound vacuole (arrowed), but as breakdown proceeds the vacuole ruptures and the sperm remnants (sr) are released into the cytoplasm. ×19,000.

Fig. 7. Part of the body wall of a 5-day-old planula larva. The body wall now consists of two epithelial layers, an inner endoderm (En) and a thicker ectoderm (Ec). The ectoderm cells bear cilia (c) and microvilli (mv) and still contain cortical granules (cg). ×8000.

The cytospines become less regularly arranged and reduce in size to resemble more typical microvilli. Sperm uptake has not been observed during post-gastrula development, and previously ingested sperm undergo breakdown. The breakdown process appears to involve the progressive decondensation of the sperm nucleus and the disintegration of the other sperm components. The surrounding vacuole membrane eventually ruptures and releases the remnants of the sperm into the cytoplasm (Fig. 6) where they disperse and apparently disappear. The cortical granules, however, persist in large numbers in the apical cytoplasm of the ectodermal cells well into larval life (Fig. 7) which, in the laboratory where settlement conditions may be poor, can extend to more than 8 weeks.

Discussion

A dramatic cortical reaction has previously been described for the sea anemone *Bunodosoma cavernata*, involving a massive discharge of cortical granules with disruption of the oolemma and cytospines, loss of mitochondria and endoplasmic reticulum, and a significant reduction in the volume of the egg (Dewel and Clark, 1974). No ultrastructural details of subsequent early development are available for this species, however. A possibly comparable process involving shedding of the outer egg layers was observed in a small proportion of uncleaved *A. fragacea* eggs during the present study. However, since the structures lost from the egg during this process were undoubtedly seen to be present in cleavage and later stage embryos, it is not thought likely that it is part of normal development in *A. fragacea*. This process might represent a stage in the breakdown of unfertilized or defective eggs in this species.

The cortical reaction is perhaps best understood for some echinoderm species, where the cortical granules discharge into the space between the oolemma and the vitelline coat immediately after fertilization. One of their functions may be to cause an elevation and toughening of the vitelline coat which helps prevent the entry of further sperm into the egg (for reviews see Schuel, 1978; Shapiro and Eddy, 1980). In *A. fragacea*, the cortical granules are apparently not discharged at fertilization, and the egg does not possess a vitelline coat. In this connection it is interesting to note that *Actinia* sperm do not have a well-defined acrosome (Larkman and Carter, 1980, and unpublished observations). A number of other instances have been reported of invertebrate eggs in which either no visible cortical reaction occurs or the cortical granules are seen to persist into later developmental stages. These include *Mytilus* (Humphreys, 1967), *Chaetopterus* (Anderson, 1974), *Urechis* (Paul, 1975; Gould-Somero and Holland, 1975), *Spisula* (Longo, 1976), and *Haliotis* (Lewis *et al.*, 1982). The cortical granules of *Hydra* eggs also remain intact after fertilization (Honegger, 1983). The role of the cortical granules in *A. fragacea* and these other species remains to be determined. Humphreys (1967) has suggested that the slow discharge of cortical granules in *Mytilus* may serve to maintain the vitelline coat during early development. In the absence of a vitelline coat, *A. fragacea* cortical granules presumably do not function in this way, and their discharged contents might suffer dispersal in the surrounding sea water.

Some means of polyspermy prevention presumably does operate soon after fertilization in *A. fragacea*. Supernumary sperm have not been found in cleavage stages, and the vast majority of embryos seem able to develop apparently normally in spite of exposure in the laboratory to sperm concentrations which may be higher than expected in the field. The nature of this mechanism is at present unknown but may be similar to the fast electrical block suggested for other species (for a review, see Dale and Monroy, 1981). It may be that this unknown mechanism operates effectively throughout cleavage but later fails and, at least under laboratory conditions, may allow the entry of large numbers of sperm into gastrula stage embryos.

By the gastrula stage, the entry of additional sperm may pose little threat to the genetic constitution of the embryo, and in *A. fragacea* the ingested sperm appear to be rapidly broken down. It is not certain that the uptake process is selective and specific for sperm; other particles of similar size might be ingested in the same way. The uptake of particles other than sperm has not been observed but, in the closed tanks in which the spawnings occurred, sperm might be the most abundant particles of suitable size available at the time. The observation that sperm were apparently not taken up by post-gastrula embryos might argue for a degree of specificity, but could also be accounted for by the reduced availability of sperm to these later stages. The ageing sperm tend to settle on the floors of the tanks, while the now motile larvae swim actively and spend less time on the bottom. However, if uptake is non-specific, it is still unclear why an early embryo which has barely begun to mobilize its own yolk reserves should be active in the uptake of particles from the external environment. The present report is based on laboratory spawnings during only a single breeding season, and its findings must be considered preliminary in nature. It is hoped that further study will shed more light on some of the interesting features of early development in this lower metazoan.

References

Anderson, E. 1974. Comparative aspects of the ultrastructure of the female gamete. *Int. Rev. Cytol.*, Suppl. 4, 1–70.

Dale, B. and Monroy, A. 1981. How is polyspermy prevented? *Gamete Res.*, 4, 151–169.

Dewel, W. C. and Clark, W. H. 1974. A fine structural investigation of surface specializations and the cortical reaction in eggs of the cnidarian *Bunodosoma cavernata*. *J. Cell Biol.*, 60, 78–91.

Gould-Somero, M. and Holland, L. 1975. Fine structural investigation of the insemination response in *Urechis caupo*. *Devl Biol.*, 46, 358–369.

Guraya, S. S. 1982. Recent progress in the structure, origin, composition and function of cortical granules in animal egg. *Int. Rev. Cytol.*, 78, 257–360.

Honegger, T. G. 1983. Ultrastructural and experimental investigations of sperm–egg interactions in fertilization of *Hydra carnea*. *Roux's Arch. Dev. Biol.*, 192, 13–20.

Humphreys, W. J. 1967. The fine structure of cortical granules in eggs and gastrulae of *Mytilus edulis*. *J. Ultrastruct. Res.*, 17, 314–326.

Larkman, A. U. 1980. Ultrastructural aspects of gametogenesis in *Actinia equina* L. In *Developmental and Cellular Biology of Coelenterates* (eds. P. Tardent and R. Tardent), pp. 61–66. Elsevier/North-Holland Biomedical Press, Amsterdam.

Larkman, A. U. 1981. An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria: Anthozoa). *Int. J. Invertebr. Reprod.*, 4, 114–167.

Larkman, A. U. and Carter, M. A. 1980. The spermatozoon of *Actinia equina* L. var *mesembryanthemum*. *J. mar. biol. Ass. U.K.*, 60, 193–204.

Lewis, C. A., Talbot, C. F. and Vacquier, V. D. 1982. A protein from abalone sperm dissolves the egg vitelline layer by a nonenzymatic mechanism. *Devl Biol.*, 92, 227–239.

Longo, F. J. 1976. Cortical changes in *Spisula* eggs upon insemination. *J. Ultrastruct. Res.*, 56, 226–232.

Paul, M. 1975. The polyspermy block in eggs of *Urechis caupo*. *Expl Cell Res.*, 90, 137–142.

Schäfer, W. G. and Schmidt, H. 1980. The anthozoan egg: differentiation of internal oocyte structure. In *Developmental and Cellular Biology of Coelenterates* (eds. P. Tardent and R. Tardent), pp. 47–52. Elsevier/North-Holland Biomedical Press, Amsterdam.

Schuel, H. 1978. Secretory functions of egg cortical granules in fertilization and development: a critical review. *Gamete Res.*, 1, 299–382.

Shapiro, B. M. and Eddy, E. M. 1980. When sperm meets egg: biochemical mechanisms of gamete interaction. *Int. Rev. Cytol.*, 66, 257–302.

J. mar. biol. Ass. U.K. (1980) 60, 193–204
Printed in Great Britain

THE SPERMATOZOON OF *ACTINIA EQUINA* L. VAR. *MESEMBRYANTHEMUM*

A. U. LARKMAN AND M. A. CARTER

Department of Biological Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth, Hants

(Figs. 1–3)

The spermatozoon of *Actinia equina* var. *mesembryanthemum* is of the primitive type, although lacking a well-defined acrosome. The sperm nucleus is cylindrical, about 1·25 μm long and 1 μm in diameter. The posterior surface of the nucleus is indented to form a shallow nuclear fossa in which lies the proximal centriole, which is inclined at an angle of approximately 45° to the long axis of the sperm. The distal centriole, which gives rise to the tail flagellum, is surrounded by a ring of nine pericentriolar processes. The anterior region of the tail is surrounded by a cytoplasmic collar, against which terminate projections from the pericentriolar processes. The midpiece of the sperm contains a single, large mitochondrial complex, positioned asymmetrically, and which is associated with a single lipid-like droplet. Numbers of clear, cored and dense vesicles are found in the midpiece, some of which resemble the 'donut' or pro-acrosomal vesicles of other species. A dense, membrane-bound body with a characteristically angular electron-lucent core of varying size and shape is also found, usually alongside the basal region of the nucleus. Its function is unknown, but it resembles the residual body-like inclusions seen in gastrodermal cells. The sexual reproduction of *Actinia equina* is imperfectly understood and appears unusual in several respects. The spermatozoon, however, appears normal and comparable to other anthozoan sperm so far examined.

INTRODUCTION

Actinia equina var. *mesembryanthemum*, the beadlet anemone (Stephenson, 1935), is a very common and widely distributed littoral anthozoan, whose sexual reproduction shows several interesting characteristics. Adult sea anemones of both sexes brood planulae and more advanced developmental stages within the gastrovascular cavity, although earlier embryonic stages are rarely found brooded in this way. Chia & Rostron (1970) suggest that embryos are expelled from the parent female anemone at an early stage and pass through a free-living phase before re-entering anemones of either sex for brooding. However, recent work (Cain, 1974) suggests that juvenile anemones are genetically related to the adult anemones in which they are brooded, and also the distribution of genetic material during sexual reproduction appears to be abnormal (Carter & Thorp, 1979). In an attempt to achieve a better understanding of the unusual sexual reproduction of this species, an ultrastructural investigation of gametogenesis was undertaken. This paper describes the fine structure of the spermatozoon within the testis.

Cnidarian spermatozoa are considered to be of the primitive type as put forward by Franzén (1956, 1970), having a short, rounded head, a midpiece containing several basically unmodified mitochondria and a tail consisting of a long flagellum. Unlike the classical primitive spermatozoon, cnidarian sperm do not possess a well-defined acrosome, although groups of Golgi-derived vesicles which may correspond to an acrosome have been described by various authors.

0025-3154/80/2828-1199 $1.20

Most of the cnidarian spermatozoa examined by electron microscopy thus far belong to the class Hydrozoa and these include *Phialidium gregarium* (Szollosi, 1964), *Eudendrium ramosum* (Summers, 1972; Hanisch, 1966; 1970), *Pennaria tiarella* (Summers, 1970), *Campanularia flexuosa*, (Lunger, 1971), *Tubularia larynx* (Afzelius, 1971; Hinsch & Clark, 1973), *Hydractinia* sp. (Hinsch & Clark, 1973) and various species of *Hydra* (Burnett, Davis & Ruffing, 1966; Shincariol, Habowsky & Winner, 1967; Weissman, Lentz & Barrnett, 1969; Stagni & Lucchi, 1970; Moore & Dixon, 1972; Zihler, 1972; West, 1978).

Scyphozoan species studied include *Cyanea* (Hinsch & Clark, 1970), *Nausithoe* (Afzelius & Franzén, 1971) and *Aurelia* (Hinsch & Clark, 1973).

Anthozoan sperm studied so far include *Metridium senile* (Hinsch & Clark, 1973) and *Bunodosoma cavernata* (Dewel & Clark, 1971, 1972; Clark & Dewel, 1974) while Lyke & Robson (1975) studied spermatid differentiation in *Protanthea simplex*, *Gonactinia prolifera*, *Calliactis parasitica* and *Parazoanthus lucificum*. A short review is given by Hinsch (1974).

We were unable to obtain naturally spawned sperm from *Actinia* adults in the laboratory, so the observations in this paper are based on the apparently most mature sperm found in the central regions of the testicular cysts. Dewel & Clark (1972) found that with *Bunodosoma* the most mature sperm found within the testicular cysts were identical in appearance to naturally spawned sperm. Chia & Spaulding (1972) and Spaulding (1974) make the point, however, that the final maturation of sea anemone sperm may occur after their release.

MATERIALS AND METHODS

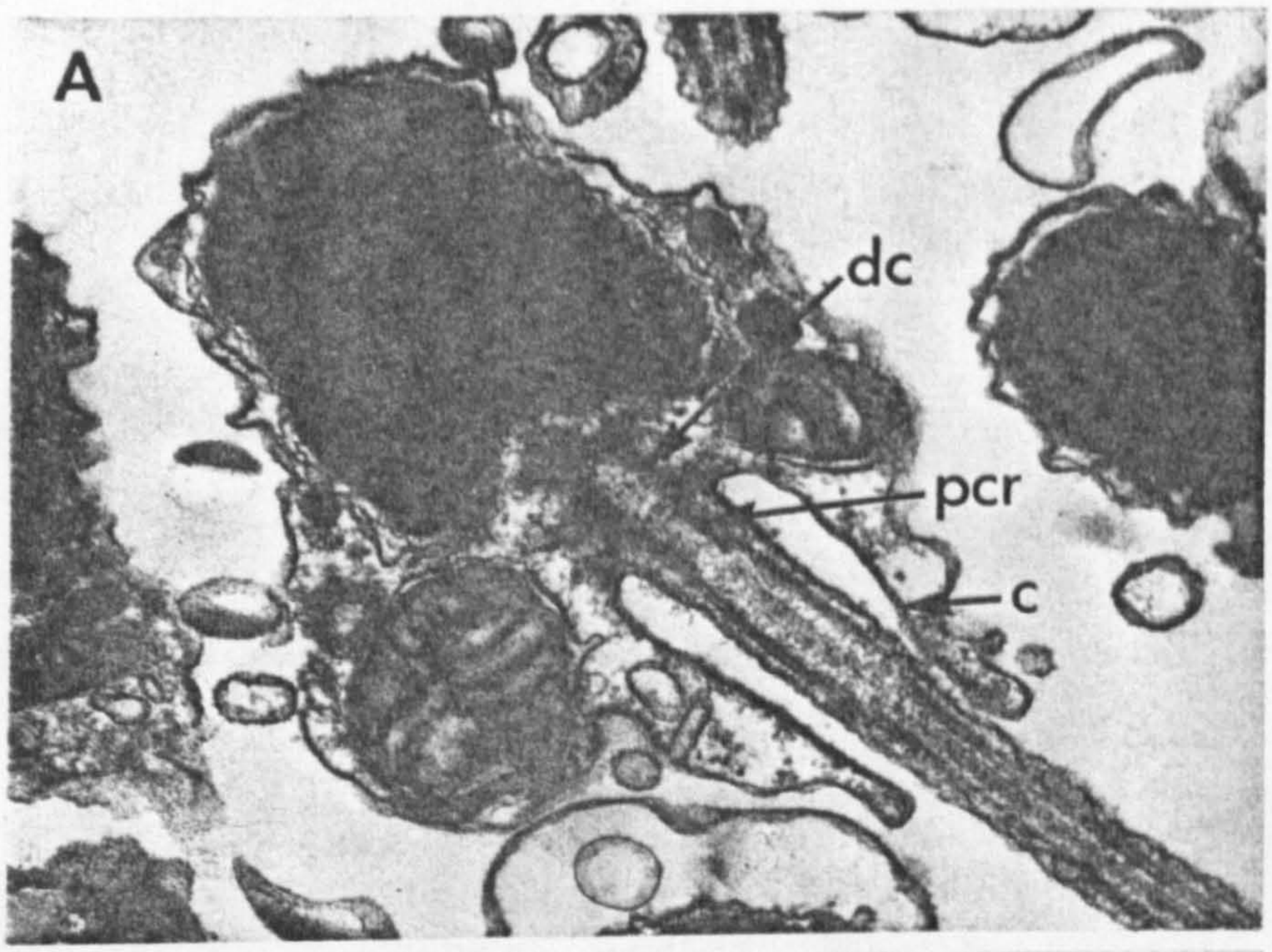
Adult specimens of *Actinia equina* var. *mesembryanthemum* were collected from the Wembury shore near Plymouth, England. Pieces of mesentery containing testicular cysts were removed and fixed in 3% glutaraldehyde in a phosphate buffered salt solution. The material was postfixed in 1% osmium tetroxide in the same buffer, and stained *en bloc* with 1·5% aqueous uranyl acetate before dehydration in graded ethanol solutions and embedding in 'EMix' epoxy resin (EMscope Laboratories Ltd, London, England). Silver-grey sections were cut using an LKB Ultrotome III ultramicrotome, double stained with uranyl acetate and lead citrate and examined using a Philips EM300 electron microscope.

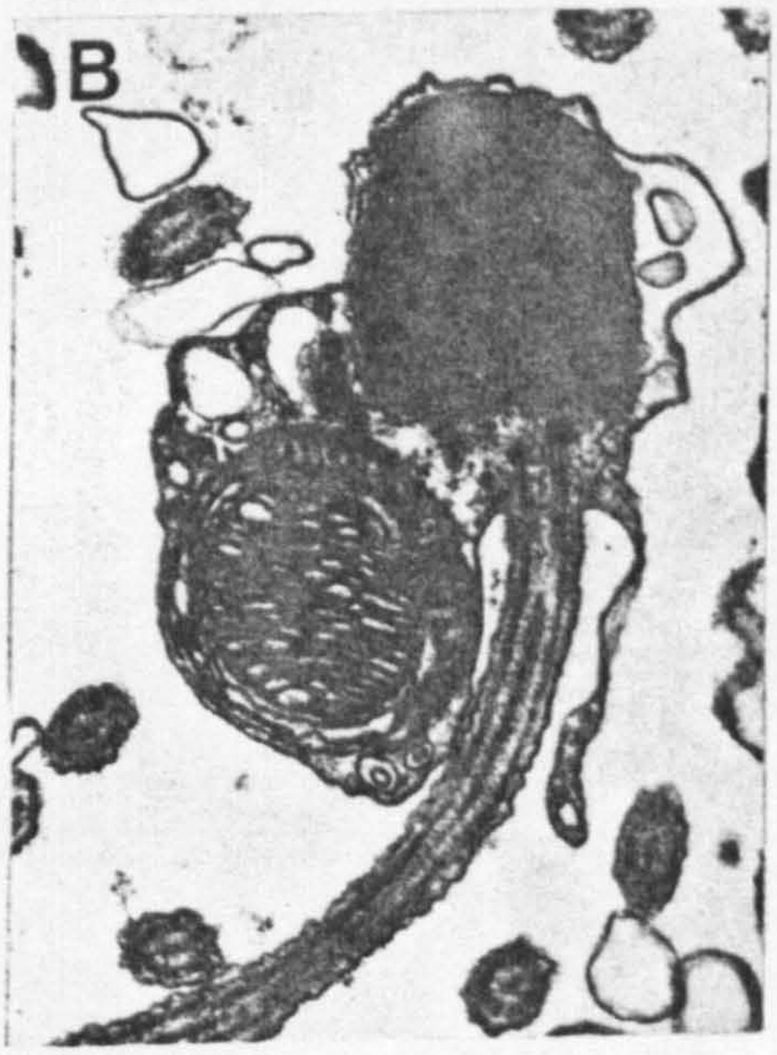
RESULTS

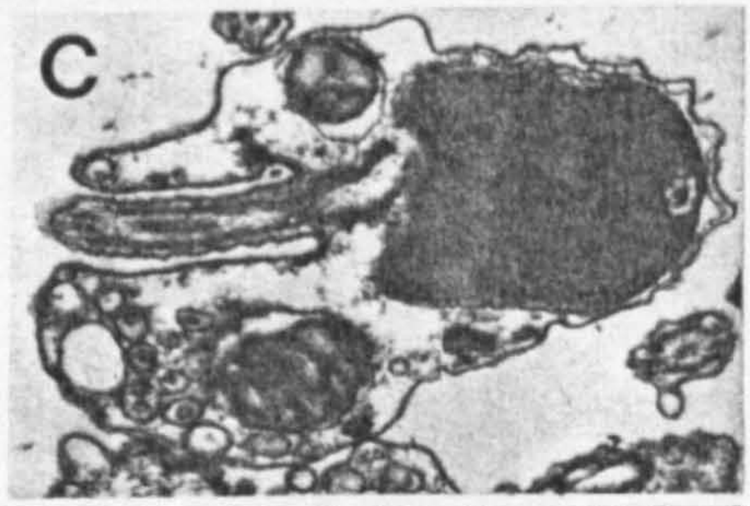
The nucleus

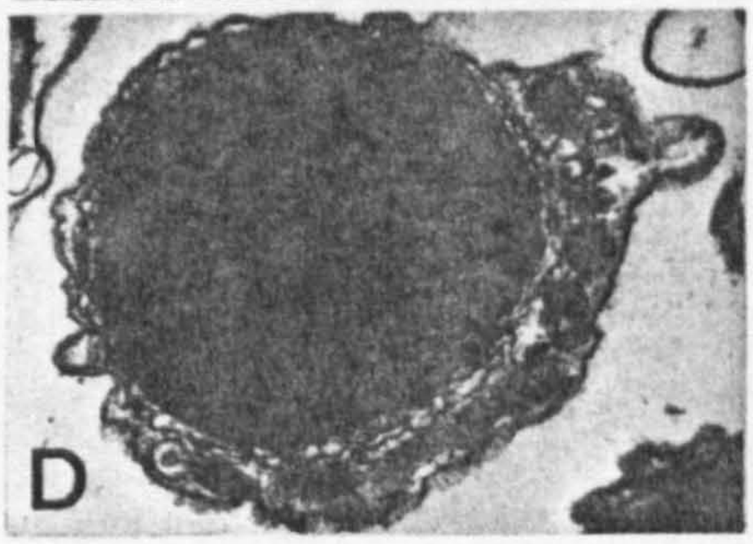
The sperm nucleus is a short cylinder of coarsely granular electron-dense material approximately 1 μm in diameter and 1·25 μm long. The anterior face of the cylinder is

Fig. 1. (A) Longitudinal section through sperm head and midpiece. c, Cytoplasmic collar; dc, distal centriole; pcr, post-centriolar region. ×40000. (B) Longitudinal section through sperm head and midpiece, showing asymmetrical position of mitochondrial complex. ×24000. (C) Longitudinal section through sperm head and midpiece. Note the clear area in the anterior region of the nucleus, and the clear and cored vesicles seen in the midpiece around the cytoplasmic collar. ×24000. (D) Transverse section through the basal region of the nucleus, showing dense vesicles arranged around the nucleus. ×37000.


A
dc
pcr
c


B


C


D

domed outwards, and the caudal surface domed inwards to form a shallow, saucer-shaped fossa (see Fig. 1A). Small electron-lucent areas some 125 nm in diameter are found within the dense nuclear material, most commonly close to the anterior and posterior surfaces of the nucleus (Fig. 1C).

The double membrane surrounding the nucleus is highly irregular and loosely fitting around the lateral surfaces, but more regular and more closely applied to the nuclear material in the region of the caudal fossa. Over the anterior surface of the nucleus, the inner membrane appears smooth and very closely applied to the nuclear material, while the outer membrane is loose and irregularly folded.

The centrioles and tail

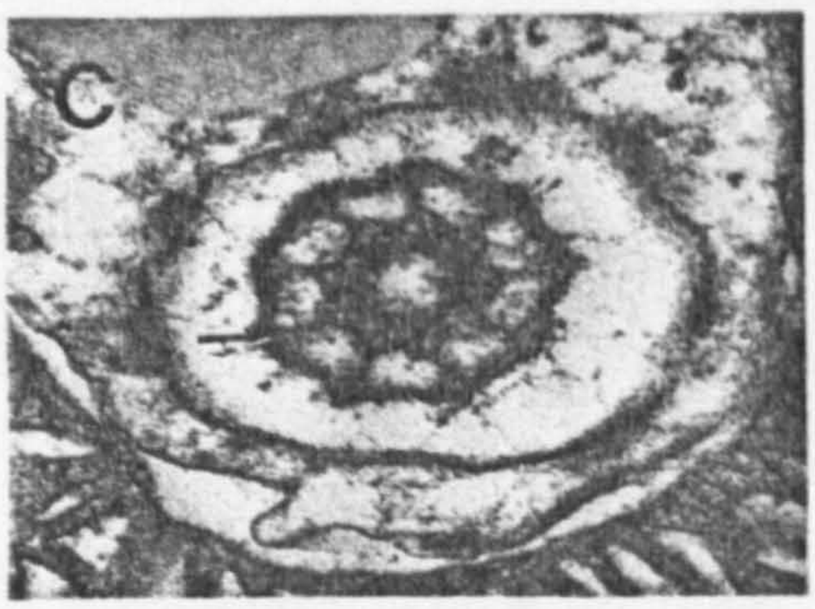
The midpiece of the sperm contains the two centrioles. The proximal centriole lies in the saucer-shaped nuclear fossa, and is aligned at an angle of 45° to the long axis of the sperm. The distal centriole gives rise to the tail flagellar axoneme (Fig. 1A). The centriole is surrounded by a ring of nine pericentriolar processes which resemble curved spokes radiating out from the centriole (Fig. 2A). The triplets of microtubules which comprise the centriole are embedded in an electron dense material which appears to be continuous with the pericentriolar processes. The tip of each pericentriolar process curves towards the posterior end of the sperm and contacts the plasma membrane of the cytoplasmic collar around the anterior region of the tail (Fig. 2D).

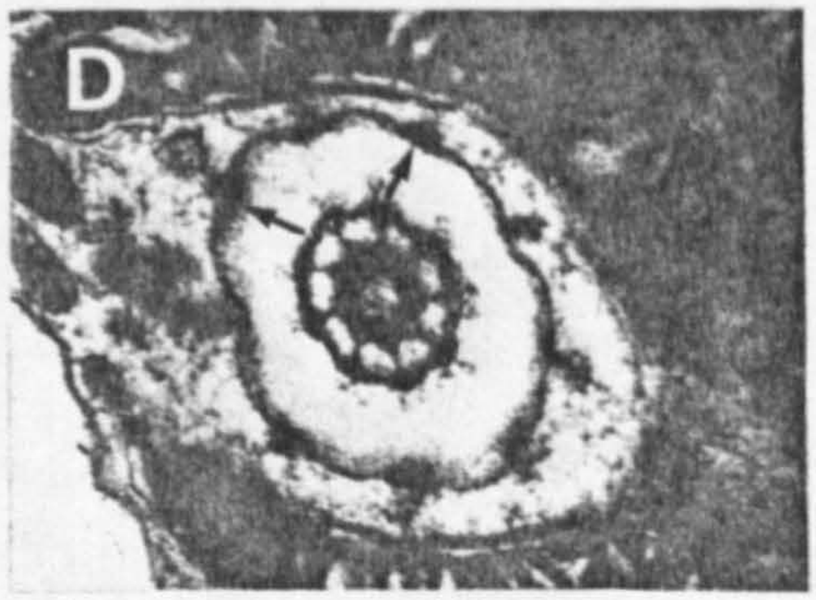
Immediately distal to the centriole is a short post-centriolar region (Fig. 2B), in which the nine tubule doublets of the tail axoneme surround a ring of 18 tubules of smaller diameter which in turn surround a ring of electron-dense material (Fig. 2C). From each outer tubule doublet arises a Y-shaped fibril which extends to the tail plasma membrane

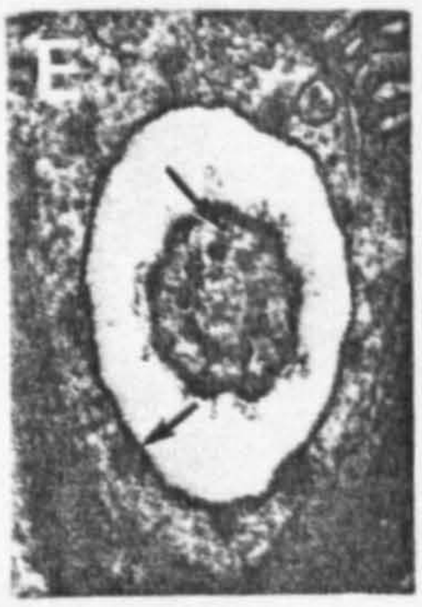
Fig. 2. (A) Transverse section through the midpiece at the level of the distal centriole. Note the ring of pericentriolar processes (arrowed) arising from the electron-dense matrix surrounding the centriolar triplet tubules, and also the arrangement of the mitochondrial complex. ×43000. (B) Transverse section through the midpiece at the level of the post-centriolar region of the tail. Note the lipid-like droplet (L) between the mitochondrial complex and the cytoplasmic collar. ×40000. (C) Enlargement of part of B, showing structure of the post-centriolar region of the tail. Note the Y-shaped fibrils (arrowed) extending from the outer tubule doublets to the tail plasma membrane and the tufts of glycocalyx-like material on the outside of that membrane. ×110000. (D) Another transverse section through the post-centriolar region of the tail, showing the nine caudal projections of the pericentriolar processes (arrowed) making contact with the plasma membrane of the cytoplasmic collar. ×57000. (E) Transverse section through tail and cytoplasmic collar immediately posterior to the postcentriolar region. Note the absence of central singlet microtubules in the tail at this level, and the Y-shaped fibrils still just visible (upper arrow). The caudal projections of the pericentriolar processes can be seen around the cytoplasmic collar membrane (lower arrow). ×61000. (F) Transverse section through the posterior region of the cytoplasmic collar. Note the typical '9+2' arrangement of microtubules in the tail and the layer of electron-dense material deposited around the cytoplasmic collar plasma membrane (arrowed). ×61000. (G) Transverse section through sperm tails showing typical '9+2' arrangement of microtubules. ×87000.


A

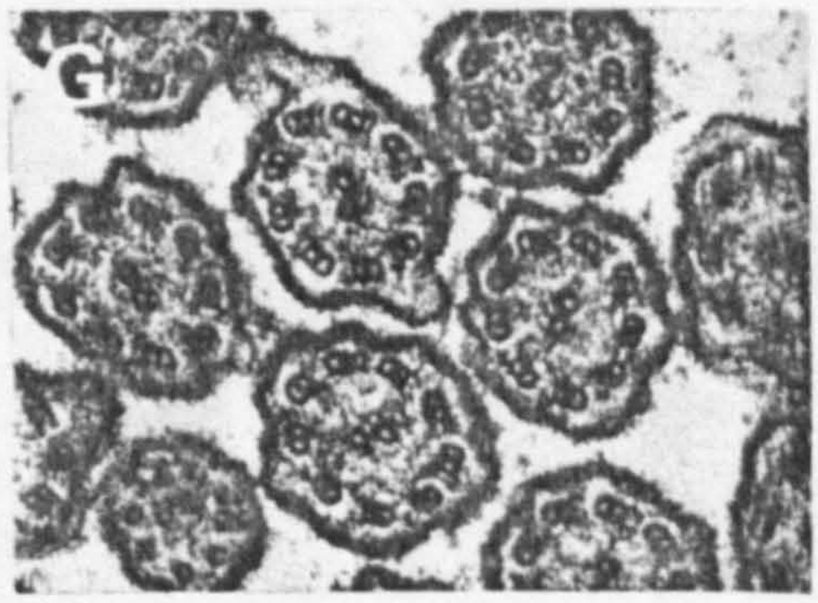

B
L


C


D


E


F


G

(arrow Fig. 2C). At some levels, the outer arms of the Y-fibrils themselves appear to bifurcate before contacting the plasma membrane. The plasma membrane is indented between the point of contact with the nine Y-fibrils, to give a scalloped appearance in cross-section, and a small tuft of finely fibrous glycocalyx-type material is found outside the plasma membrane at each point of contact (Fig. 2C).

Distal to the post-centriolar region, the tail axoneme consists of only a ring of nine tubule doublets for a distance of about 200 nm. In some cases, the Y-fibrils found in the neck region can still be seen arising from the tubule doublets in this region (Fig. 2E). Distally the two central singlet tubules appear, and the bulk of the tail has a typical flagellar 9+2 arrangement of microtubules (Fig. 2G).

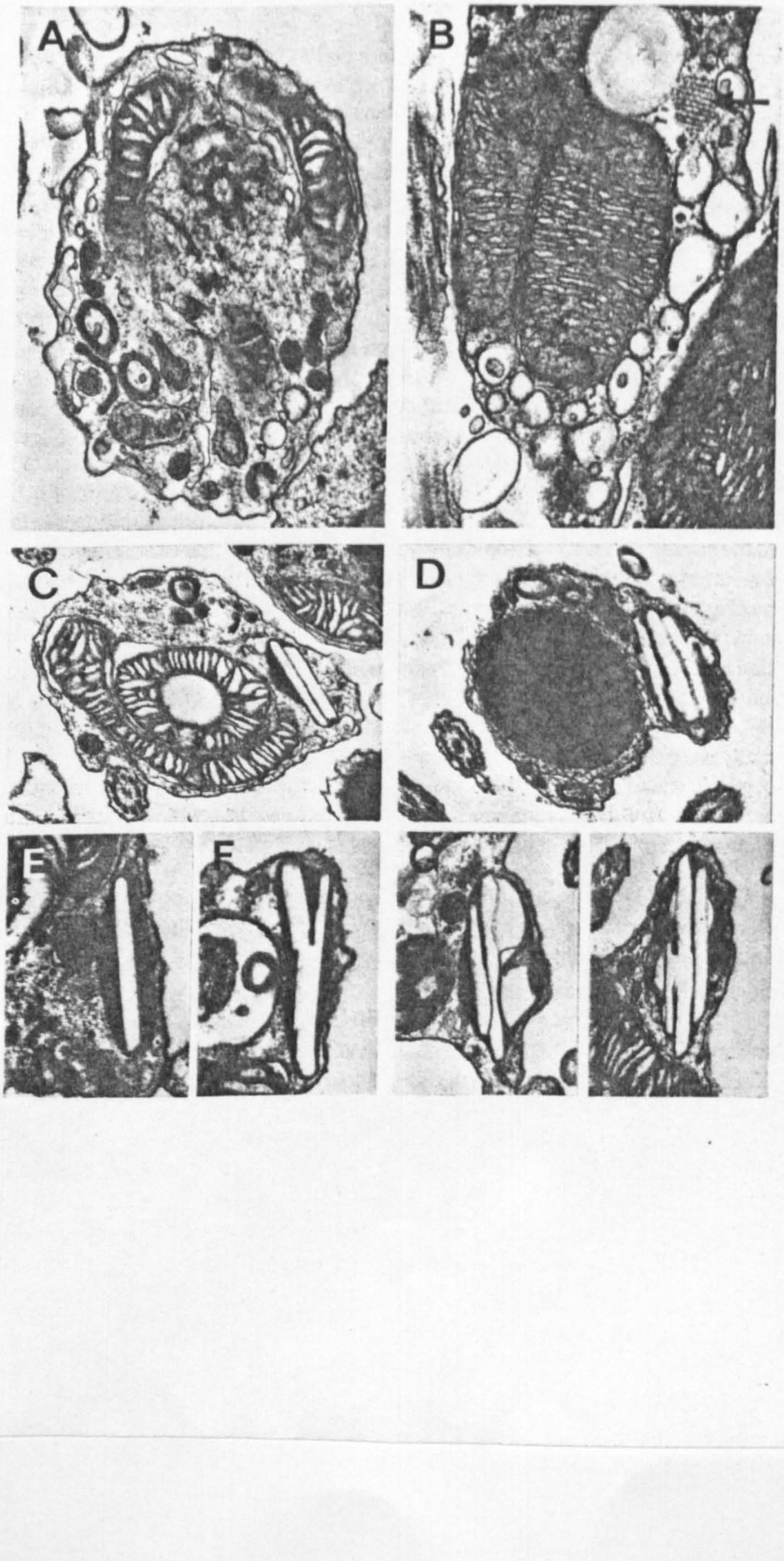
The anterior region of the tail is surrounded by a deep infolding of the sperm plasma membrane which forms a cytoplasmic collar. The collar extends from the junction of the distal centriole with the neck region of the tail posteriorly for about 1 μm. Lying in the cytoplasm just inside the plasma membrane of the collar there is a layer of electron-dense material about 15 nm from the membrane, which may serve to increase the rigidity of the collar structure (Fig. 2F).

The tail plasma membrane has an irregular, wavy appearance both in transverse and longitudinal section. The tail tapers towards the tip and the axoneme tubules are progressively lost, so that tail profiles containing various numbers and arrangements of tubules are seen. The extreme tip appears to be devoid of tubules.

Mitochondrial complex

The midpiece of the sperm contains a single complex mitochondrion, which is positioned asymmetrically to the mid-line of the sperm (Fig. 1B). The form of the mitochondrion appears to vary from one sperm to another. In some cases it consists of a spherical core enveloped by lateral extensions or processes from the core, similar to that described for *Bunodosoma*. In others it appears to consist of a slender mitochondrial sheet coiled or folded on itself. The mitochondrial complex is always closely associated with, and sometimes completely surrounds, a single droplet of lipid-like material some 400 μm in diameter (Fig. 3C).

Fig. 3. (A) Oblique section through sperm midpiece. Note the number and variety of vesicles seen in the lower half of the picture. ×40000. (B) Oblique section through sperm midpiece. Note the lipid-like droplet, closely associated with the mitochondrial complex and the clear and cored vesicles. A crystalline body is arrowed in the top right-hand corner of the picture. ×54000. (C) Oblique section through sperm midpiece, showing the mitochondrial complex surrounding a lipid-like droplet. Note the residual body-like inclusion. ×25000. (D) Transverse section through basal region of nucleus. Note 'donut' vesicles and residual body-like inclusion around nucleus. ×31000. (E–H) Various forms of residual body-like inclusions. E, F ×47000; G, H ×33000.


A
B
C
D
E
F

Vesicles

Several different types of membrane-bound vesicle are found irregularly scattered through the posterior regions of the sperm head. No vesicles are found around the anterior region of the nucleus.

The most common form of vesicle is about 120 nm in diameter and contains material of low electron density, often surrounding a membrane-bound core of slightly greater electron density. These vesicles are normally found in the posterior regions of the midpiece and seem especially numerous in the cytoplasmic collar around the anterior portion of the tail (Fig. 3B).

In the more anterior parts of the midpiece, and around the base of the nucleus, are found dense vesicles of various shapes (Fig. 3A). The most characteristic type of dense vesicle is elongate and in section may be curved into a horseshoe shape or further curved into a ring. The electron-dense contents are not homogeneous but have a fibrillar structure. These vesicles resemble the 'donut' or 'pro-acrosomal' vesicles described for other species. Other dense vesicles appear roughly sperhical and contain a homogeneous, highly electron-dense material.

Residual body

In many sections of sperm, a single large dense body, with a characteristic straight-sided electron-lucent core is found, usually in the midpiece or lateral to the caudal part of the nucleus (Fig. 3D). The body is bounded by a trilaminar membrane, and varies greatly in size and appearance (Fig. 3E–H). The dense component varies in electron-density and is sometimes homogeneous but often contains membrane remnants and whorls. The electron-lucent core may be rectangular or other more complex shapes, and there may be several cores of various sizes and shapes in the same body. Similar structures are very commonly seen in the gastrodermal cells of this species, apparently associated with phagocytic vacuoles, and have been described as 'residual bodies' in gastrodermal cells of other species (see Discussion).

Crystalline body

A small spherical body, not bounded by a plasma membrane, composed of dense particles arranged in a regular array reminiscent of a crystalline lattice is sometimes seen in sections of the midpiece, usually associated with the lipid-like droplet (Fig. 3B).

DISCUSSION

The squat cylindrical shape of the nucleus of *Actinia* sperm is unusual when compared with the elongate cone or wedge shape found in most hydrozoan and scyphozoan sperm, and in the anthozoans *Calliactis*, *Protanthea* and *Gonactinia* (Lyke & Robson, 1975) and *Metridium* (Hinsch & Clark, 1973). The nucleus of *Parazoanthus* sperm is spherical (Lyke & Robson, 1975), while that of *Bunodosoma* (Dewel & Clark, 1972) is dome-shaped and more similar to that of *Actinia*. The irregularity of the nuclear membrane, especially along the lateral surfaces, is a common feature among cnidarian sperm, as is the presence of small electron-lucent areas within the nuclear material, although the significance of these is unclear.

All cnidarian sperm studied so far show some form of pericentriolar apparatus surrounding the distal centriole. The most complex arrangements seem to be found among the Hydrozoa and Scyphozoa, perhaps most notably in the scyphozoan *Nausithoe* (Afzelius & Franzén, 1971), while the more modest ring of unbranched pericentriolar processes seen in *Actinia* seems similar to those seen in other Anthozoa. The fact that in *Actinia*, and possibly others, these processes terminate on the plasma membrane of the cytoplasmic collar, in a region where that membrane is apparently stiffened by an electron-dense layer deposited inside it, may argue for a structural anchoring function for the pericentriolar apparatus.

While the pericentriolar processes arise from the electron-dense material surrounding the centriole and emerge through the spaces between the tubule triplets, the Y-fibrils of the post-centriolar region immediately distal to the centriole arise from the tubule doublets themselves. We feel this may warrant their consideration as separate structures, possibly with a separate function, rather than as mere distal continuations of the pericentriolar processes.

All anthozoan sperm studied, except *Parazoanthus*, possess a cytoplasmic collar around the anterior region of the tail flagellum. That seen in *Actinia* is well developed and complete, while those of *Bunodosoma* and *Calliactis* show interruptions by small regions of fusion between the tail and collar membranes. The function of the collar is unknown, but in *Actinia*, at least, it seems to form part of a rigid, integrated structure with the distal centriole and the pericentriolar apparatus, which may serve as a firm anchorage for the tail.

The large asymmetrical mitochondrial complex is presumably the product of fusion of several smaller mitochondria during spermiogenesis. Similar complexes have been described for *Bunodosoma* and *Metridium*, while most cnidarian sperm midpieces contain a ring of often 4–6 mitochondria, although a degree of asymmetry may be introduced by one mitochondrion being larger than the others, as in *Tubularia* (Afzelius, 1971). The advantage of an asymmetrical arrangement to a presumably actively swimming cell is unclear, although, as Lyke & Robson (1975) point out, it may affect the position of the centre of gravity of the sperm which might tend to stabilize or orientate the sperm body during swimming. Lipid-like droplets are seen in many anthozoan sperm and presumably act as high-energy food reserves. The close relationship between the droplet and the mitochondrial complex seen in *Actinia* would seem sensible if this were the case. Additionally the droplet may act as a buoyancy aid which could also play a role in stabilizing the sperm during locomotion.

Dense 'donut' vesicles are consistently found in cnidarian sperm. In hydrozoan and scyphozoan sperm they are often found anterior to the nucleus, in the position usually occupied by the acrosome in other sperm. The 'donut' vesicles are reported to be of Golgi origin, as is the acrosome of other sperm, and in the hydrozoan *Campanularia* they have been shown to undergo changes when the sperm is in contact with the female reproductive tissue. It is thus suggested that these vesicles may be an evolutionary forerunner of the acrosome, and may perform a similar function, and are often referred to as 'proacrosomal' vesicles. In Anthozoa, however, vesicles of this type are not found anterior to the nucleus, but rather distributed laterally around its base. In *Actinia* a large number of

vesicles of various types are found in the sperm midpiece, and while some of those found in the most anterior regions of the midpiece are reminiscent of 'donut' vesicles, they are very variable in size and shape. Some of the other types of vesicle might represent earlier stages in the maturation of the characteristic 'donut' vesicle or may represent a heterogeneous population of vesicles with various functions. Until the role, if any, of these vesicles at fertilization is observed, we feel that any attempt to ascribe a function to any of them may be unwise.

The residual body-like structures found in *Actinia* sperm have not, as far as we are aware, been observed in any other cnidarian sperm (or sperm from any other group). We have consistently observed similar, although much larger, bodies associated with phagocytic vacuoles in gastrodermal cells in *Actinia*. Chapman (1974) describes similar structures, which he terms residual body-like inclusions, in both ectodermal and endodermal cells of *Aurelia aurita*, and Lentz (1966) suggests that similar structures, which he terms crystalloid bodies, may be observed in digestive cells of many cnidarians. In *Actinia* sperm, these bodies vary considerably in size and form from sperm to sperm, which makes it difficult to propose a specific function for them. However, if they are related to the structures seen in digestive cells, they may be supposed to contain digested or partly digested food material, and hydrolytic enzymes. They might thus act as a food reserve, or enzymes within them might conceivably play some role at fertilization. Alternatively, these bodies may represent the breakdown products of excess cytoplasm and organelles from earlier spermatogenic stages, and might be expelled from the sperm after release from the testicular cyst. Work is in progress to try to localize hydrolytic enzyme activity within these bodies.

Crystalline bodies have been reported in sperm from several groups (e.g. Phillips, 1970), although not previously from Cnidaria. In these cases, the bodies are normally closely associated with mitochondria, and may represent a highly condensed form of mitochondrial material. In *Actinia* sperm, they do not seem to be particularly closely associated with the mitochondrial complex. Crystalline bodies were seen only rarely in our sections, and their significance is unclear.

Of the anthozoan sperm thus far described that of *Actinia equina* var. *mesembryanthemum* is most similar in overall configuration to the sperm of *Bunodosoma cavernata*. Both have a short, blunt nucleus and show a high degree of asymmetry caused by the lateral location of the single large mitochondrial complex. *Metridium* sperm also have a single mitochondrial complex, but show a lesser degree of asymmetry and have an elongate wedge-shaped nucleus. The sperm of *Calliactis*, *Protanthea* and *Gonactinia* are less like that of *Actinia* and have elongate, wedge-shaped nuclei, and a relatively symmetrical ring of several mitochondria showing varying degrees of fusion. The most dissimilar sperm of *Parazoanthus* is unusual, having a spherical body containing a spherical nucleus, a loosely arranged ring of dumpy mitochondria and with no cytoplasmic collar. As sperm from more species of Anthozoa are examined by electron microscopy, it will be interesting to see if they can be grouped together according to their basic structural plan and whether their groupings can be related to an evolutionary sequence or perhaps some feature of their ecology or reproductive behaviour.

Preliminary genetical studies have indicated that this variety of *Actinia equina* has an

unusual method of sexual reproduction in which, in the absence of parental selection of young, the nuclear fusion component of fertilization may not occur or may occur only rarely (Carter & Thorp, 1979). The results described in this paper suggest that the spermatozoon of this variety is comparable with those previously described for other species of Anthozoa, and give no indication that the sperm are degenerate or abnormal.

REFERENCES

Afzelius, B. A., 1971. Fine structure of the spermatozoon of *Tubularia larynx* (Hydrozoa, Coelenterata). *Journal of Ultrastructure Research*, 37, 679–689.

Afzelius, B. A. & Franzén, A., 1971. The spermatozoon of the jelly-fish *Nausithoe*. *Journal of Ultrastructure Research*, 37, 186–199.

Burnett, A. L., Davis, L. E. & Ruffing, F. E., 1966. A histological and ultrastructural study of germinal differentiation of interstitial cells arising from gland cells in *Hydra viridis*. *Journal of Morphology*, 120, 1–8.

Cain, A. J., 1974. Breeding system of a sessile animal. *Nature, London*, 247, 289–290.

Carter, M. A. & Thorp, C. H., 1979. The reproduction of *Actinia equina* L. var. *mesembryanthemum*. *Journal of the Marine Biological Association of the United Kingdom*, 59, 989–1001.

Chapman, D. M., 1974. Cnidarian histology. In *Coelenterate Biology, Reviews and New Perspectives* (ed. L. Muscatine and H. M. Lenhoff), pp. 2–92. New York: Academic Press.

Chia, F. S. & Rostron, M. A., 1970. Some aspects of the reproductive biology of *Actinia equina* (Cnidaria: Anthozoa). *Journal of the Marine Biological Association of the United Kingdom*, 50, 253–264.

Chia, F. S. & Spaulding, J. G., 1972. Development and juvenile growth of the sea anemone *Tealia crassicornis*. *Biological Bulletin. Marine Biological Laboratory, Woods Hole, Mass.*, 142, 206–218.

Clark, W. H. & Dewel, W. C., 1974. The structure of the gonads, gametogenesis and sperm-egg interactions in the Anthozoa. *American Zoologist*, 14, 495–510.

Dewel, W. C. & Clark, W. H., 1971. The fine structure of the spermatozoon of *Bunodosoma* sp. (Cnidaria). *Biology of Reproduction*, 5, 86 [abstract].

Dewel, W. C. & Clark, W. H., 1972. An ultrastructural investigation of spermatogenesis and the mature sperm in the anthozoan *Bunodosoma cavernata* (Cnidaria). *Journal of Ultrastructure Research*, 40, 417–431.

Franzén, A., 1956. On spermiogenesis, morphology of the spermatozoon, and the biology of fertilization among invertebrates. *Zoologiska bidrag frdn Uppsala*, 31, 355–482.

Franzén, A., 1970. Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. In *Comparative Spermatology* (ed. B. Baccetti), pp. 24–46. New York: Academic Press.

Hanisch, J., 1966. Spermienentwicklung von *Eudendrium ramosum*. *Naturwissenschaften*, 53, 587.

Hanisch, J., 1970. Die Blastostyl- und spermienentwicklung von *Eudendrium racemosum* Cavolini. *Zoologische Jahrbücher* (Abteilungen für Anatomie und Ontogenie der Tiere), 87, 1–62.

Hinsch, G. W., 1974. Comparative ultrastructure of cnidarian sperm. *American Zoologist*, 14, 457–465.

Hinsch, G. W. & Clark, W. H., 1970. Comparative studies of coelenterate sperms. *Journal of Cell Biology*, 47, 88a [abstract].

Hinsch, G. W. & Clark, W. H., 1973. Comparative fine structure of cnidarian spermatozoa. *Biology of Reproduction*, 8, 62–73.

Lentz, T. L., 1966. *The Cell Biology of Hydra*. x, 199 pp. Amsterdam: North-Holland.

Lunger, P. D., 1971. Early stages of spermatozoon development in the colonial hydroid *Campanularia flexuosa*. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, 116, 37–51.

Lyke, E. B. & Robson, E. A., 1975. Spermatogenesis in Anthozoa; differentiation of the spermatid. *Cell and Tissue Research*, 157, 185–205.

Moore, G. P. M. & Dixon, K. E., 1972. A light and electron microscopical study of spermatogenesis in *Hydra caulicaulata*. *Journal of Morphology*, 137, 483–502.

Phillips, D. M., 1970. Insect sperm: their structure and morphogenesis. *Journal of Cell Biology*, 44, 243–277.

SCHINCARIOL, A. L., HABOWSKY, J. E. J. & WINNER, G., 1967. Cytology and ultrastructure of differentiating interstitial cells in spermatogenesis in *Hydra fusca*. *Canadian Journal of Zoology*, 45, 590–593.

SPAULDING, J. G., 1974. Embryonic and larval development in sea anemones (Anthozoa: Actiniaria). *American Zoologist*, 14, 511–520.

STAGNI, A. & LUCCHI, M. L., 1970. Ultrastructural observations on the spermatogenesis in *Hydra attenuata*. In *Comparative Spermatology* (ed. B. Baccetti), pp. 357–361. New York: Academic Press.

STEPHENSON, T. A., 1935. *The British Sea Anemones*, vol. II. 426 pp. London: The Ray Society.

SUMMERS, R. G., 1970. The fine structure of the spermatozoon of *Pennaria tiarella* (Coelenterata). *Journal of Morphology*, 131, 117–129.

SUMMERS, R. G., 1972. An ultrastructural study of the spermatozoon of *Eudendrium ramosum*. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, 132, 147–166.

SZOLLOSI, D., 1964. The structure and function of centrioles and their satellites in the jellyfish *Phialidium gregarium*. *Journal of Cell Biology*, 21, 465–479.

WEISSMAN, A., LENTZ, T. L. & BARRNETT, R. J., 1969. Fine structural observations on nuclear maturation during spermiogenesis in *Hydra littoralis*. *Journal of Morphology*, 128, 229–240.

WEST, D. L., 1978. Ultrastructural and cytochemical aspects of spermiogenesis in *Hydra hymanae*, with reference to factors involved in sperm head shaping. *Developmental Biology*, 65, 139–154.

ZIHLER, J., 1972. Gametogenese und Befruchtungsbiologie von *Hydra*. *Wilhelm Roux Archiv für Entwicklungsmechanik Organismen*, 169, 239–277.

Developmental and Cellular Biology of Coelenterates
P. Tardent and R. Tardent, editors

ULTRASTRUCTURAL ASPECTS OF GAMETOGENESIS IN *Actinia equina* L.

ALAN LARKMAN
Department of Biological Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth, England.

INTRODUCTION

Actinia equina, the common beadlet sea anemone, exists in two varieties, the more common variety *mesembryanthemum* and the rarer strawberry form, variety *fragacea*. The var. *fragacea* is dioecious and oviparous, while *mesembryanthemum* broods its young within the gastrovascular cavity[1]. Young are found brooded by *mesembryanthemum* adults with gonads of both sexes as well as individuals without gonads, and the origin of these broods has been the subject of considerable interest. Chia and Rostron[2] suggested that young might be released from their parents at an early stage and might enter adults of either sex for brooding. Other possibilities have also been considered, including phasic hermaphroditism, parthenogenesis[3] and a selection/cloning process[4], but none seems entirely satisfactory. It was thus felt that an ultrastructural study of gametogenesis in the two varieties might be of interest.

Our knowledge of sea anemone gametogenesis is as yet only fragmentary. Sperm from a number of species have been described at the ultrastructural level (see Lyke and Robson[5] and a review by Hinsch[6]). Aspects of oocyte structure have been briefly described by Spaulding[7], Clark and Dewel[8] and Dewel and Clark[9], but no comprehensive account has yet been published. The present paper represents a survey of work in progress rather than an exhaustive treatment of any particular aspect. Many of the findings, especially those concerning the early stages of spermatogenesis and oogenesis, must be regarded as preliminary.

MATERIALS AND METHODS

Large individuals of both varieties were collected at intervals throughout the year from the rocky intertidal zone at Wembury, near Plymouth. Small pieces of gonad were fixed by immersion in 3% glutaraldehyde in a phosphate buffered salt solution, rinsed in buffer, trimmed further if required and post-fixed in 1% osmium tetroxide in the same buffer. The pieces were then dehydrated, and embedded in EMix epoxy resin (EMscope Labs. Ltd., London). Sections were cut on an LKB Ultratome III and stained with uranyl acetate and lead citrate before examination using a Philips EM300 electron microscope.

RESULTS

The histological appearance of the gonads of both varieties is identical, and there are few differences at the ultrastructural level. The following account therefore applies to both varieties, except where stated. There is, however, a difference in the frequency of occurrence of gonads. Most individuals of the var. *fragacea* possess well developed gonads throughout the year, while only about 25% of *mesembryanthemum* individuals were found to have gonads. The sexes appear to be separate.

The male gonad

The spermatogenic cells are found in roughly spherical testicular cysts in the mesogloea of the mesenteries, between the gastric filament and the mesenteric retractor muscle. The cells arise among the bases of the gastrodermal cells and migrate into the mesogloea, where they aggregate and proliferate. As the cyst enlarges, later spermatogenic stages are seen accumulating in the centre of the cyst. Eventually the outer layer of spermatogonia and spermatocytes is depleted and the entire cyst is filled with spermatozoa. The cyst is generally completely enclosed by a thin sheet of mesogloeal material, and mesogloeal cells are often found flattened around its periphery. At one point on the surface of the mature cyst, specialized gastrodermal cell bases penetrate the mesogloeal layer and make contact with the interior of the cyst. In this region, non-germinal and apparently phagocytic cells are found, possibly derived from the gastroderm.

Spermatogenesis

The spermatogonia are rounded, 5-8 µm in diameter with large nuclei. The nucleus contains a single large nucleolus, which is usually situated against the nuclear membrane. The scant cytoplasm contains few organelles. Tail structures are often seen, consisting of two centrioles at right angles to each other, one of which gives rise to the tail axoneme. There appear to be no consistent ultrastructural differences between spermatogonia and primary spermatocytes. The latter can only be identified when their nuclei are seen to contain synaptinemal complexes. Cells apparently undergoing meiosis are often seen. In these cells the nuclear envelope is fragmentary or absent and the chromosomal material appears as irregular shaped dense granular masses occupying most of the cell. The details of meiosis are as yet unclear, but cytokinesis is incomplete, and the result is a group of four spermatids linked by narrow cytoplasmic bridges. During spermateleosis, the mitochondria become larger and fewer in number, presumably by fusion. Nuclear condensation involves the

migration of nuclear material to an area between two dense plaques which form on opposite sides of the nuclear envelope.

Spermatozoa

The mature sperm nucleus is cylindrical in shape with a domed anterior surface, and contains a dense granular material. The single mitochondrial complex is positioned to one side of the long axis of the sperm giving it an ungainly, asymmetrical appearance. The distal centriole is surrounded by a ring of nine spoke-like pericentriolar processes and behind the distal centriole a Y-shaped fibril radiates from each microtubular doublet to contact the tail membrane. The proximal region of the tail is surrounded by a cytoplasmic collar. The sperm body also contains a single large lipid droplet, and numerous vesicles, some of which resemble the pro-acrosomal vesicles described in other coelenterate species.

The Female Gonad

Oocytes develop in the mesogloea of the mesenteries in the same position as do the testicular cysts in the male. The mature oocyte is roughly spherical and about 200 µm in diameter. The nucleus or germinal vesicle is large and contains a single nucleolus which may be 10 µm in diamter and contains numerous small dense granules which may be ribosomes. The nuclear envelope is perforated by numerous pores. Around the nucleus is a band of rough endoplasmic reticulum, which in places may consist of more than 40 parallel lamellae. Stacks of annulate lamellae are common amongst the E.R.

The ooplasm contains numerous yolk granules and lipid droplets. Two major classes of yolk granule may be distinguished, although other types are also found.

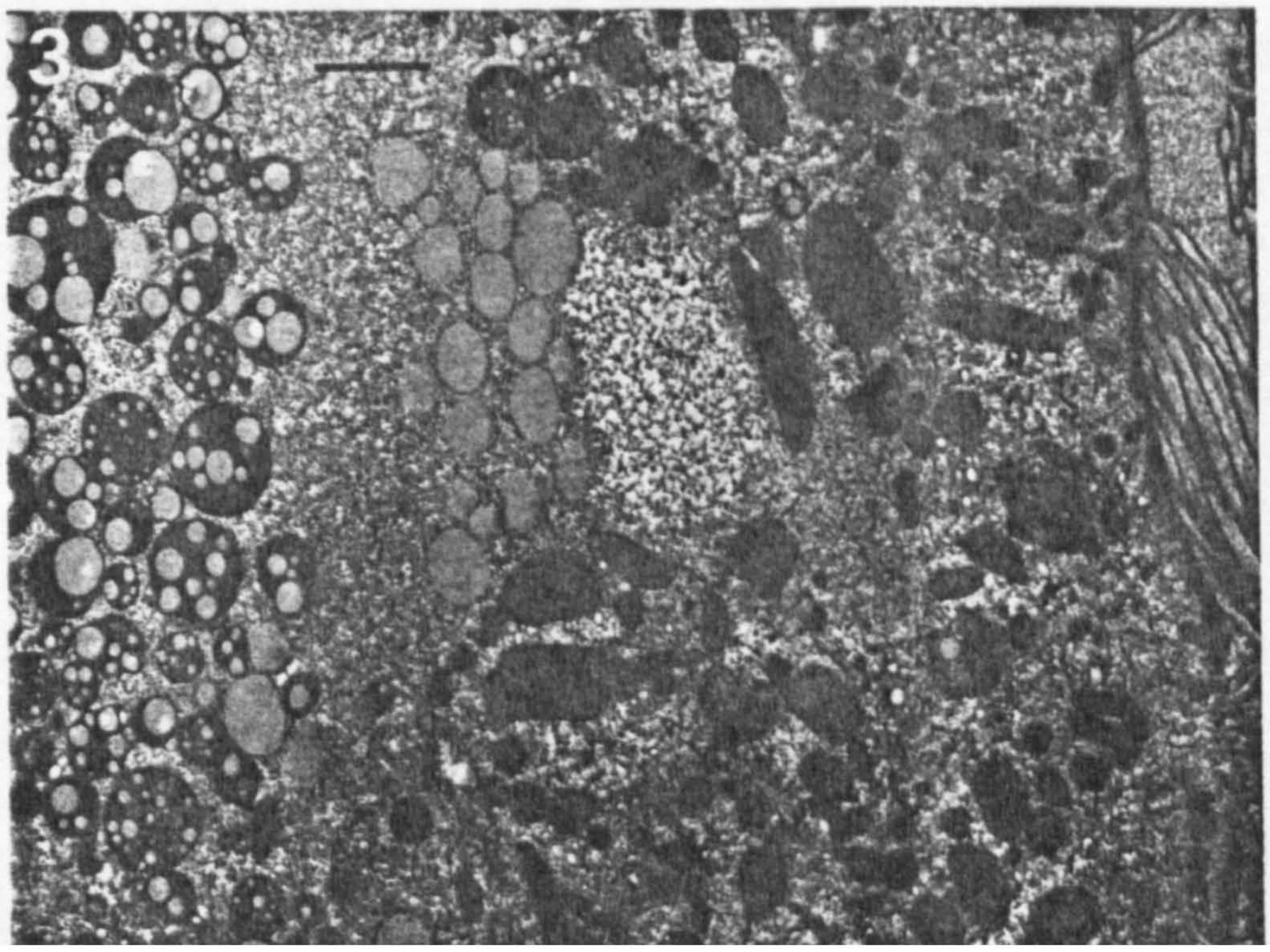
Type I - compound yolk granules. These consist of a number of electron lucent inclusions embedded in an electron dense matrix, which may also contain membrane fragments.

Type II - Fibrillar yolk granules. These are bundles of regularly arranged parallel fibrilswithin a limiting membrane. These yolk granules are abundant in *fragacea* oocytes, but are not found in *mesembryanthemum*.

Numerous Golgi complexes and accumulations of endoplasmic reticulum are found throughout the ooplasm. In the peripheral regions of the oocyte are found many cortical granules containing a dense homogeneous material. The surface of the oocyte bears tufts of cytospines or macrovilli, which normally lie folded flat against the oocyte. Each cytospine contains many dense fibrils, which extend from the base of the cytospine as a rootlet. Each oocyte is


1


2


3

enclosed by a thin mesogloeal layer which isolates it from other oocytes and mesenteric cells, except in two cases:-

1 Mesogloeal cells are found flattened closely against the surface of the oocyte, within the mesogloeal covering, often filling the spaces between tufts of cytospines.

2 In one specific region, facing the outside of the ovary, specialized gastrodermal cell bases penetrate the mesogloeal covering and spread out into a depression in the oocyte surface. Such a structure was described by Dunn[10] in *Epiactis* ovaries and termed a trophonema.

Early Stages

Oocytes of various sizes are often found together in the same mesentery. In light microscope sections, cells which are thought to be oogonia or very small oocytes are found among the bases of mesenteric gastrodermal cells, where they may be distinguished by their spherical shape and intense basophilia. It is thought that these cells then migrate into the mesogloea, but they have not yet been examined with the electron microscope. The smallest cells recognizable as oocytes which have been seen with the E.M. are about 20 µm in diamter, and already have a large nucleus and a prominent nucleolus. Their cytoplasm contains endoplasmic reticulum and ribosomes, but no yolk granules. In the variety *fragacea*, fibrillar yolk granules appear long before any others.

DISCUSSION

There has long been the suggestion that the two varieties of *Actinia equina* should be regarded as separate species[1], and recent enzyme studies[11] have lent support to this view. It might also be expected that the different reproductive strategies of the two varieties might be reflected in differences in gamete morphology. However, the only difference between the two so far discovered is the total absence of fibrillar yolk granules from the *mesembryanthemum* oocyte, and the lower incidence of gonads in that variety. It may be that the brooding variety, which is also the more common form, needs to direct less of its metabolic resources towards gamete production.

Explanation of plate.
Fig. 1. Group of spermatids, linked by cytoplasmic bridges. Bar = 1 µm.
Fig. 2. Mature spermatozoon, showing asymmetric location of mitochondrial complex. Bar = 0.5 µm.
Fig. 3. Low power micrograph of oocyte cytoplasm, showing from left to right, compound yolk granules, lipid droplets, fibrillar yolk granules, dense cortical granules and cytospines at the oocyte surface. Bar = 2 µm.

The sperm of *Actinia* is similar to that of *Bunodosoma cavernata*[12] although both differ markedly from several other anthozoan sperm[5,6]. Oogenesis in *Actinia* appears to follow the pattern described for *Epiactis* by Dunn[10]. This process is in marked contrast to that seen in *Hydra*[13], and marine colonial hydroids[14], in which the oocyte enlarges by engulfment and fusion with surrounding oocytes. The abundance of endoplasmic reticulum and Golgi complexes in the *Actinia* oocyte, and the lack of obvious uptake activity at the oocyte surface, suggests that the bulk of yolk synthesis takes place within the oocyte. Yolk granules have not been described in detail for any other sea anemone, but *Actinia* compound yolk granules resemble those described in *Bunodosoma* oocytes[9], and the fibrillar granules resemble structures shown in a micrograph of *Peachia* oocytes by Spaulding[7].

Some of the present observations appear to be at variance with the earlier findings of Chia and Rostron[2], working with the variety *mesembryanthemum*. They found synchronous development of oocytes within a single mesentery, while I found oocytes at various stages of development intermingled, in line with the findings of Dunn for *Epiactis* and Spaulding for *Peachia*. Additionally, Chia and Rostron found sperm developing in elongated tubules, reminiscent of vertebrate seminiferous tubules, while I found that sperm developed in roughly spherical testicular cysts, similar to those found in *Bunodosoma*[12]. Local and seasonal variation may help to explain these apparent anomalies.

REFERENCES

1. Stephenson, T. A. (1935) The British Sea Anemones. The Ray Society, London, pp. 1-426.
2. Chia, F. S. and Rostron, M. A. (1970) J. mar. biol. Ass. U.K., 50, 253-264.
3. Gashout, S. E. and Ormond, R. F. G. (1979) J. mar. biol. Ass. U.K. (in press).
4. Carter, M. A. and Thorp, C. H. (1979) J. mdr. biol. Ass. U.K. (in press).
5. Lyke, E. B. and Robson, E. A. (1975) Cell Tiss. Res., 157, 185-205.
6. Hinsch, G. W. (1974) Amer. Zool., 14, 457-465.
7. Spaulding, J. G. (1974) Amer. Zool., 14, 511-520.
8. Clark, W. H. and Dewel, W. C. (1974) Amer. Zool., 14, 495-510.
9. Dewel, W. C. and Clark, W. H. (1974) J. Cell. Biol., 60, 78-91.
10. Dunn, D. F. (1975) Biol. Bull., 148, 199-218.
11. Thorpe, J. P. and Carter, M. A. (in prep.).
12. Dewel, W. C. and Clark, W. H. (1972) J. Ultrastruct. Res., 40, 417-431.
13. Zihler, J. (1972) Wilhelm Roux Arch, Entwickl. Mech.Org., 169 239-267.
14. Van de Vyver, G. (1967). Arch. Biol. (Leige), 78, 451-518.

International Journal of Invertebrate Reproduction, 4 (1981) 147–167
Elsevier/North-Holland Biomedical Press

An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria; Anthozoa)

Alan U. Larkman

Department of Biological Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth, Hants., U.K.

(Received 6 March 1981)

Individuals from a population of the intertidal sea anemone *Actinia fragacea* (Tugwell) were collected at approximately monthly intervals over an 18 month period. Samples of gonad were removed from each anemone and examined by light and electron microscopy. During late spring and early summer, large numbers of small cells were seen in the endoderm of the female gonads, lying close to the mesoglea. For convenience, these cells were classified into three types. Type I cells are 6–9 μm in diameter, with relatively very large nuclei, which may contain synaptinemal complexes, and scant cytoplasm containing few organelles. Type II cells are larger, reaching 15 μm in diameter, with more abundant cytoplasm containing more organelles and inclusions. The nucleus is more dense, but may also contain synaptinemal complexes. Type III cells are less common. They are similar in size to Type II cells, but their nuclei contain irregular dense chromatin masses, and the nuclear envelope is incomplete or absent. The possible significance of the various cell types is discussed. It is suggested that Type I cells are oocytes at a very early stage of differentiation and that Type II cells are rather later oocytes. The status of the Type III cells is uncertain.

oocytes; ultrastructure; Anthozoa; *Actinia*

Introduction

Anthozoan gametogenesis has received increasing attention in recent years. Much recent work has been directed towards the male gametogenic process and in particular the ultrastructure of the spermatozoon [1–5] (for a review of earlier work see Ref. 6). Oogenesis and the morphology of the female gamete, however, have received less detailed study. The presumed status of coelenterates as lower metazoans makes a knowledge of their oogenesis of interest for comparative purposes. Many anthozoans are relatively large and can provide gonad material in quantity, which makes some species at least suitable subjects for detailed gametogenic studies.

Male and female gametogenic cycles have recently been investigated by light microscopy in several anthozoan species. These include the sea anemones *Epiactis prolifera* [7], *Anthopleura elegantissima* [8] and the corals *Stylophora pistillata* [9, 10] and *Astrangia danae* [11]. The approach adopted in these studies has been to take samples from the field at regular intervals throughout the year. In this way all the

0165-1269/81/0000–0000/$02.75

various stages of gametogenesis are available for examination. The limited resolution of the light microscope precludes detailed analysis of the cytological processes involved, however.

Such ultrastructural studies of anthozoan oogenesis as have been published have tended to deal with specific aspects of oocyte structure, such as the oocyte surface [12], the cortical reaction [13] or features of potential taxonomic usefulness [14,15]. No coherent description of oogenesis at the ultrastructural level has yet appeared for any anthozoan species. This lack is most clearly apparent when considering the early stages of oogenesis when the small size of the cells involved makes investigation by light microscopy difficult.

A study was therefore undertaken which combined the use of electron microscopy with regular sampling from the field. Gonad samples were taken at approximately monthly intervals from a population of the sea anemone *Actinia fragacea* (Tugwell) and the ultrastructural features of all stages of oogenesis were examined. This present paper describes the ultrastructural appearance of cells which are believed to be oocytes at the earliest stages of differentiation. Corresponding cells have been mentioned in previous studies by Dunn [7] and Jennison [8], but their small size prevented detailed description and made identification of cell types and stages difficult with the light microscope.

Actinia fragacea, the species used in this study, was until recently considered to be a variety of the species *Actinia equina*, the common beadlet anemone. Carter and Thorpe [16] proposed that the two were separate species, and their classification has been followed in this paper. *A. fragacea* was preferred to the more common *A. equina* for this study for two reasons. Firstly, although the reproduction of *A. equina* has been intensively investigated, it is still poorly understood [17–20] and may be more specialized than that of *A. fragacea*, which is thought to be oviparous, cross-fertilizing and non-brooding [21]. Secondly, it is known that a high proportion of large *A. fragacea* individuals possess recognizable gonads throughout the year, while less than 25% of sympatric *A. equina* individuals have gonads [16, 22]. Since it is not possible to establish the presence or absence of gonads in these anemones without dissection, the use of *A. fragacea* has greatly reduced the number of anemones which have had to be taken from the field. This was an important consideration for the present study since it involved repeated sampling over a long period of time from a single population.

A light microscope description of the later stages of oogenesis in *A. equina* was given by Chia and Rostron [17], and preliminary evidence of an annual cycle of gametogenic activity was provided for both *A. equina* and *A. fragacea* by Carter and Funnell [22]. A preliminary ultrastructural account of some aspects of oogenesis in *A. fragacea* was given by Larkman [23].

Materials and Methods

Samples consisting of five or six large individuals of *Actinia fragacea* (Tugwell) were collected from a restricted area of rocky shore at Wembury, near Plymouth

(Devon, England), at approximately monthly intervals between March 1979 and September 1980. The anemones were opened by making two cuts at right angles to each other across the pedal disk, and were then pinned out, pedal disk uppermost, under sea water. The gonads were then clearly visible on the mesenteries.

Small pieces of gonad were removed and fixed for electron microscopy by immersion in 3% glutaraldehyde in 0.1 M phosphate buffer containing 3% NaCl. After initial fixation for 2 h the pieces were rinsed in the same buffer and after further trimming were post-fixed for 1 h in 1% OsO_4 in the same buffer. The tissue was then dehydrated through a graded series of ethanol solutions and embedded in epoxy resin ('EMix', EMscope Labs. Ltd., London). Areas of interest were located by light microscope examination of 1-μm sections stained with toluidine blue. Ultrathin sections were cut with an LKB Ultratome III using glass or diamond knives. Sections were collected on uncoated copper grids, electron stained with uranyl acetate and lead citrate before examination using a Philips EM300 electron microscope.

Larger pieces of gonad intended solely for light microscopy were fixed in glutaraldehyde as above, partially dehydrated using ethanol and embedded in JB4 plastic resin (Polysciences Inc.). Sections 1–2 μm thick were cut with glass knives using an LKB Pyramitone, and briefly stained with 1%toluidine blue in 1% sodium tetraborate solution.

Results

Light microscope observations

The sexes in *Actinia fragacea* appear to be separate, in that all the individuals sampled contained either all male or all female gametes. The gonads, like those of other sea anemones [24, 25], are located on the mesenteries and lie between the mesenteric retractor muscles and the gastric filaments. The gonads are composed of two layers of endodermal epithelial cells sandwiching a thin layer of mesoglea. In most Anthozoa, germ cells arise in the endodermal cell layers and migrate into the mesoglea at an early stage of their development [24]. In *A. fragacea*, most oocytes were seen to enter the mesoglea in the period from June to August, and to develop there before being spawned or resorbed during the same period the following year.

Small oocytes were seen as rounded cells with relatively very large nuclei with usually a single prominent nucleolus and strongly basophilic cytoplasm. The smallest cells of this type which could be readily distinguished with the light microscope were about 15 μm in diameter, and were found among the bases of the endodermal epithelial cells, lying close to the mesoglea of the gonad. Cells of this type were found occasionally through the entire gonad region during most of the year, especially in samples from June to October. However, in gonads sampled during June and July, additional areas containing very much greater numbers of this type of cell were seen. These areas occurred towards the edge of the gonad, close to its junction with the mesenteric retractor muscle. In these areas small oocytes (15–25 μm diameter) were so numerous as to form an almost complete band lining the mesoglea. Some could be seen apparently in the process of entering the mesoglea.

Electron microscope observations

Pieces of gonad from all the monthly samples were examined by electron microscopy. As a result of light microscopic studies, initial detailed searches for small oocytes concentrated on the June and July samples, and in particular on the gonad/retractor muscle border. Corresponding regions of gonad material sampled in May were also examined in detail.

In these areas from samples taken from May to July, large numbers of small cells (5–15 μm in diameter) with relatively large nuclei and densely staining cytoplasm were found among the bases of the endodermal cells, as well as larger (15–25 μm) oocytes. These cells had not previously been identified by light microscopy. Similar cells were subsequently found in other regions of the gonad and in samples from other times of year, but in very reduced numbers.

These small cells, not easily distinguished in light microscope sections, are the subject of the rest of this report. On the basis of their size and ultrastructural appearance they have been divided into three broad categories.

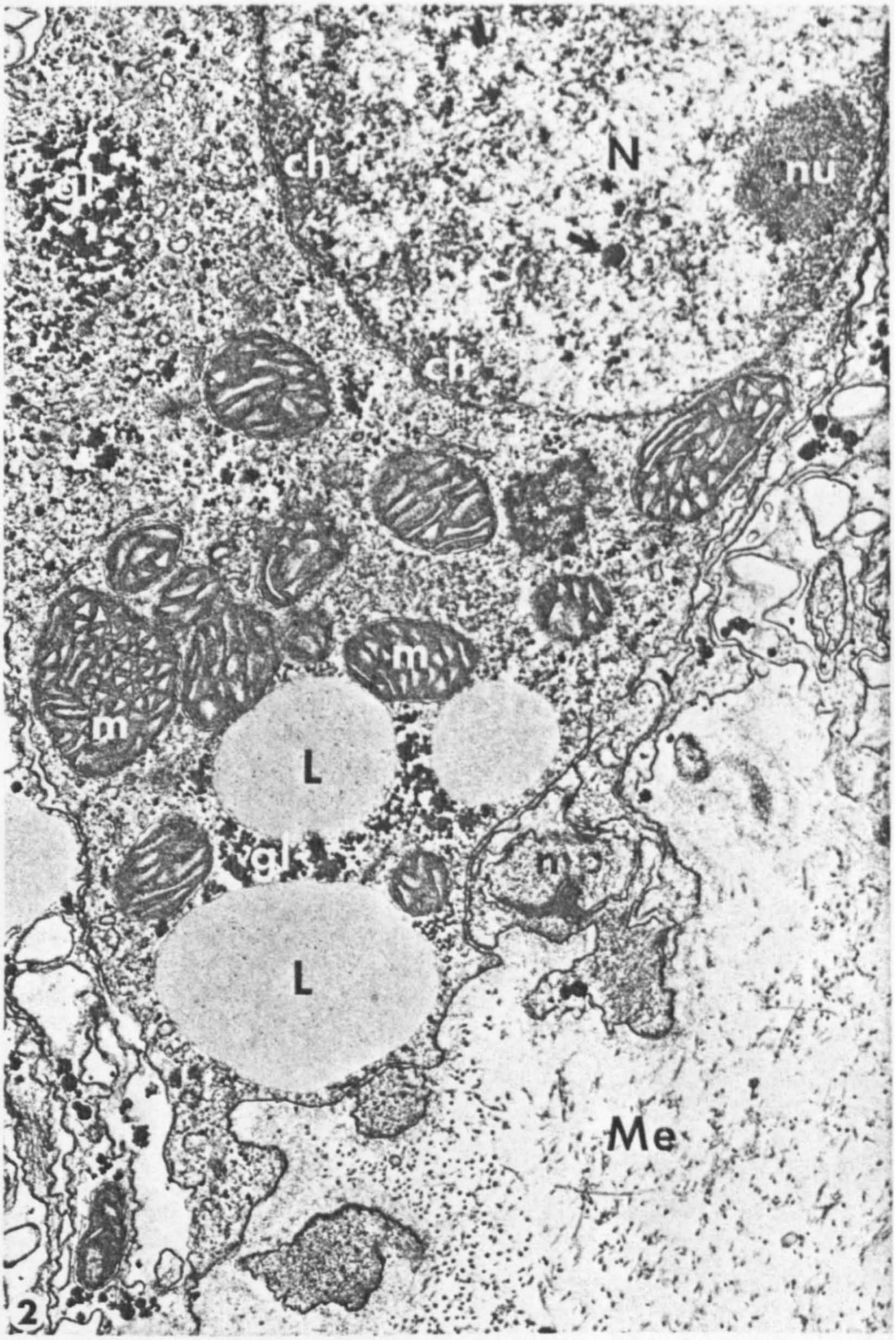
Type I cells

These are the smallest of the three cell types, and a typical cell of this type is shown in Fig. 1. These cells are 5–9 μm in diameter and are usually slightly elongate. The nucleus is large relative to the size of the cell, usually 4–6 μm in diameter, and is of generally low electron density, especially when compared to the densely staining cytoplasm. The nucleus is bounded by a well-defined nuclear envelope containing occasional nuclear pores. The chromatin is seen as irregular electron dense masses separated by areas of low electron density which in turn consist of a finely filamentous material arranged in a loosely reticular fashion through an electron lucent matrix. The dense chromatin areas often make contact with the nuclear envelope and appear to attach to it by the formation of dense plaques (Figs. 2 and 5). A single nucleolus is sometimes seen in section, always closely adpressed to the nuclear envelope, and consisting of a coarsely granular material (Figs. 2 and 4). In the nuclei of many cells of this type, characteristic three-layered synaptinemal complexes can be seen (Fig. 3). These complexes consist of two outer dense granular areas lying either side of a less dense, more finely granular central lamina. They are often seen to make contact with the nuclear envelope. Only rather short lengths of this type of complex have been seen in cells of this type. Longer lengths of otherwise identical appearance have been observed in spermatocytes in the male gonads of this species however.

Granules of dense chromatin material are sometimes seen in nuclei of cells of this type (Fig. 2). These granules are roughly spherical and average about 100 nm in diameter. Rarely, the nuclei are seen to contain one or more vesicles, up to 600 nm

Fig. 1. A small Type I cell, about 6 μm long, among gonad epithelial cell bases (Ep) and their muscle processes (mp) next to the mesoglea (Me). Note the relatively large nucleus (N) containing material of low electron density, the Golgi complex (g), mitochondria (m) and an area containing various small clear and cored vesicles (v). ×21000.

1


ch
N
nu
ch
m
m
L
gl
mp
L
Me
2

in diameter, full of small dense particles, as shown in Fig. 5. These vesicles are bounded by an irregular membrane which does not appear to be of the trilaminar unit membrane type. This membrane appears to link up with fibrils of the less dense fibrillar nuclear material. The enclosed particles appear spherical and are some 20 nm in diameter. Their true nature is uncertain.

The cytoplasm appears dense and contains numerous free ribosomes and rather few other organelles. The nucleus is often located eccentrically in the cytoplasm, with most of the organelles contained in a cytoplasmic lobe on one side of the nucleus. The cytoplasm is normally seen to contain a number of mitochondria, a Golgi complex, glycogen deposits, lipid droplets, occasional dense bodies, small vesicles of various kinds, and a flagellar basal-body–rootlet complex. The general features of many of these are shown in Fig. 2.

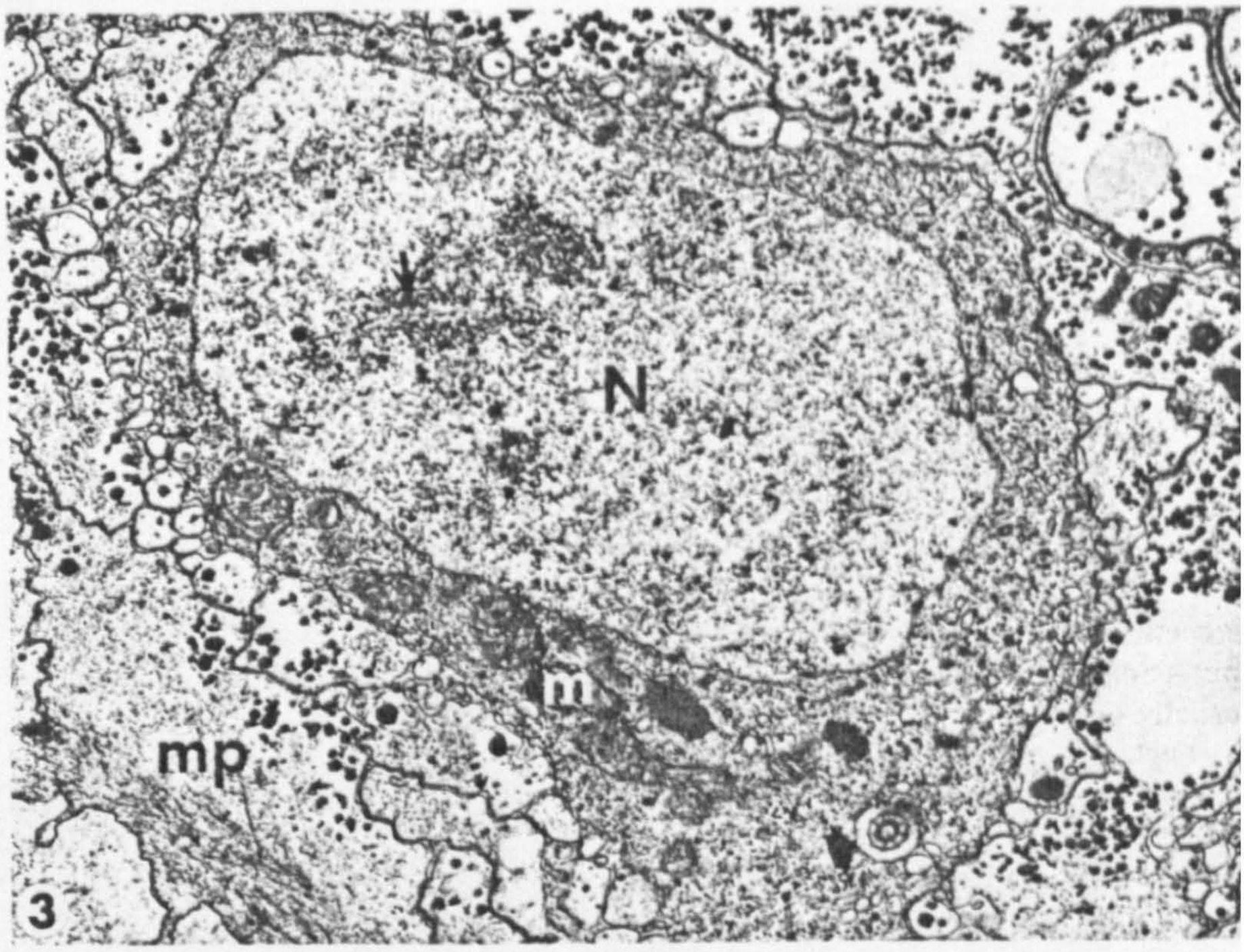
The mitochondria are small and usually squat, averaging about 400 nm in diameter and 700 nm in length. Occasionally elongate mitochondria of similar diameter but up to 1.5 μm long are observed. The cristae are numerous, and are sometimes arranged to form a characteristic lattice pattern, as shown in Fig. 2. The mitochondrial matrix is homogeneous and electron dense. The mitochondria are usually distributed to form one or two loose groups rather than scattered evenly throughout the cytoplasm.

The Golgi complex is usually situated close to the nuclear envelope, and consists of a stack of 5–7 parallel cisternae containing an electron dense material (Figs. 1 and 6). The density of the contained material often increases from one side of the stack to the other. The stack is often associated with a concentration of small vesicles and short membranous elements, all containing material of similar density and appearance to that found in the cisternae. Usually only a single complex is seen per cell in section.

Glycogen is found in small deposits scattered throughout the cytoplasm, and often associated with lipid droplets (Fig. 2). The smaller deposits contain glycogen in the β-conformation, with an average particle size of about 20 nm. Larger deposits may include a few typical rosettes of glycogen in the α-conformation, between 70 and 120 nm in diameter. The gonad epithelial cell bases often contain large amounts of glycogen (Figs. 3 and 4), but this is always of the α-conformation and the particle size is usually much larger.

One or more lipid droplets are frequently seen in the cytoplasm of Type I cells (Figs. 2 and 6). The droplets are roughly spherical and range between 0.6 and 1.2 μm in diameter. They are homogeneous and weakly electron-dense. Larger droplets of otherwise similar appearance are found abundantly in the bases of the gonad epithelial cells.

Fig. 2. Part of a Type I cell showing many characteristic features. The nucleus (N) contains a nucleolus (nu) and areas of dense chromatin (ch) next to the nuclear envelope. A chromatin granule (arrowed) can also be seen. The cytoplasm contains lipid droplets (L), glycogen (gl), and a honeycomb aggregation of dense material (asterisk). The mitochondria (m) show a characteristic lattice-like arrangement of cristae. ×28000.


N
m
mp
3

nu
m
N
gl
mp
Me
4

Various small membrane bound vesicles are frequently seen. The largest contain a homogeneous electron dense material and average 500 nm in diameter. Sometimes these dense vesicles contain small lipid-like inclusions. The most numerous vesicles are smaller, about 150 nm in diameter, and enclose a clear electron lucid interior. Some similar small vesicles also contain a single membrane-bound core of moderately dense material (Fig. 1).

Most Type I cells show evidence of the possession of a single flagellum (Figs. 3 and 4). The flagellum arises from a basal-body–rootlet complex, which consists of two centrioles at right angles to each other connected to a long striated rootlet extending into the cell. Microtubules can be seen radiating from the centrioles of the complex. An apparently identical structure is found in coral planula cells, and has been described in some detail [26,27], so a full description will not be given here. In Type I cells, the complex is situated at the base of a deep invagination of the cell surface, so that for the first part of its length, the flagellum is surrounded by a collar of cytoplasm (Figs. 3 and 4). Near its base, the flagellar axoneme has a complex structure. The nine microtubular doublets surround an inner ring of eighteen tubules, and are themselves connected to the flagellar plasma membrane by nine Y-shaped fibrils. A similar arrangement is found in the sperm of several sea anemones, including that of *Actinia* (see Discussion).

In many Type I cells, one or two aggregations of finely granular dense material are seen in the cytoplasm (Figs. 2 and 7). Rarely, this material appears as an irregular shapeless mass, but it usually has a quite distinctive honeycomb appearance as shown in Fig. 7. These aggregations consist of a number of closely packed cylinders of electron dense material, appearing as rings in transverse section. The cylinders are some 150 nm in diameter and as many as twenty conjoined rings have been seen in one section, although between three and six rings are more common. Within each ring is seen a smaller, less well defined ring which appears to be made up of particles of only moderate electron density.

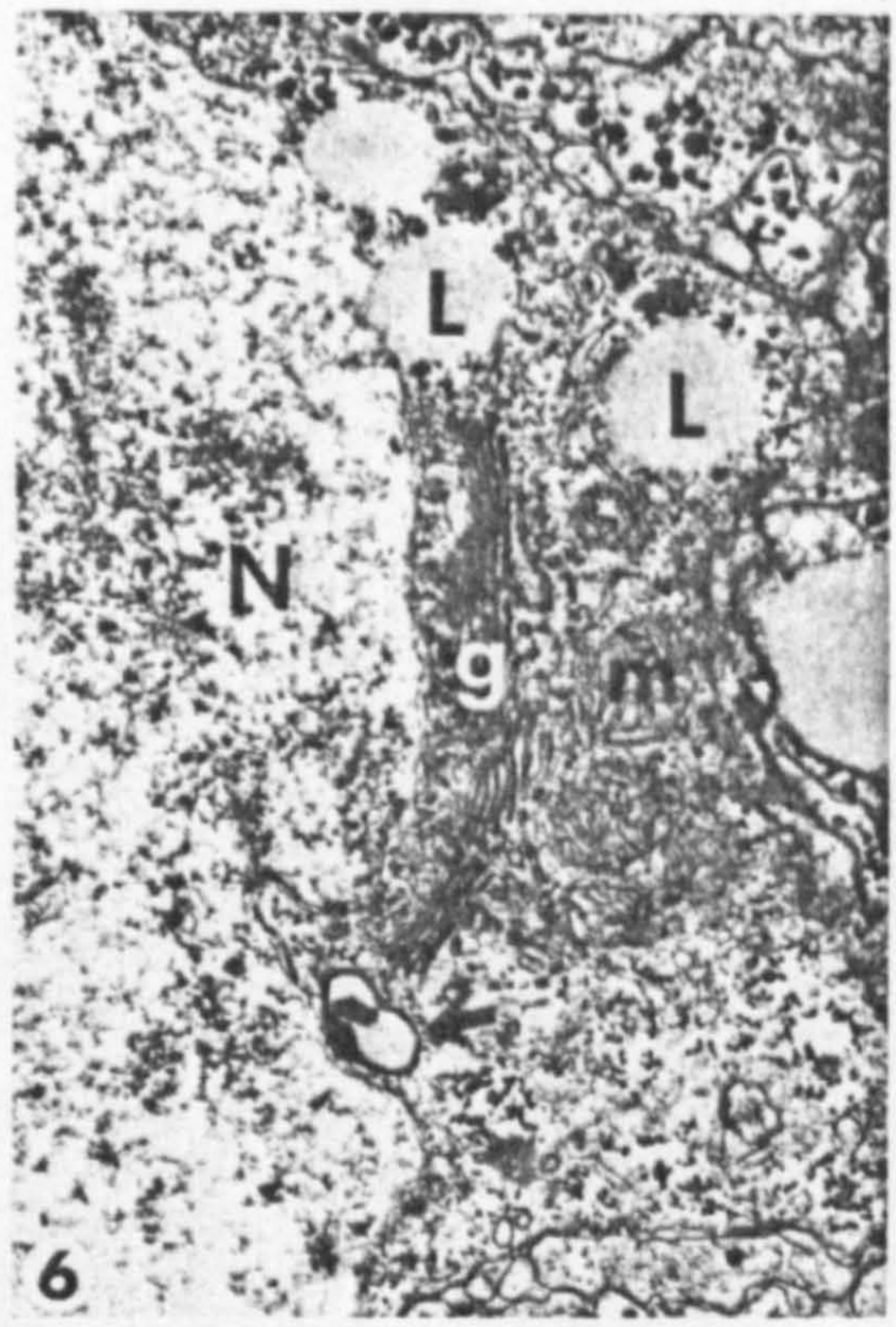
These aggregations are always seen in close association with a group of mitochondria and are usually found close to the nuclear envelope. However, they have not been observed to make contact with these or any other organelles. Identical aggregations of dense material have been seen, often more extensively developed, in spermatogonia in developing spermaries in male *A. fragacea* gonads, but have not, as yet, been seen in other cell types (unpublished observations).

Most Type I cells are seen to make contact with the mesoglea of the gonad. Many

Fig. 3. Type I cell (largest diameter 7.2 μm). The nucleus (N) contains a synaptinemal complex (arrowed), while the cytoplasm shows few organelles other than mitochondria. A flagellum (squat arrow) sectioned transversely can be seen surrounded by cytoplasm. ×15000.

Fig. 4. Type I cell, about 7 μm in diameter. The nucleus (N) contains a single nucleolus (nu) lying against the nuclear envelope. A flagellum, sectioned obliquely, is seen lying in a deep invagination in the cytoplasm (arrowed), with microtubules (arrowhead) radiating from its base. The cell is separated from the mesoglea (Me) by endodermal muscle processes (mp). Some endodermal cell bases contain extensive deposits of glycogen (gl). ×14000.

5



6

7

8

lie at the mesoglea/endoderm border and contact the mesoglea over a broad area, as in Fig. 1, while others are situated deeper in the endoderm and contact the mesoglea only by a fine cytoplasmic process, as in Fig. 8. Other cells which do not appear to contact the mesoglea may of course do so out of the plane of section.

Type I cells have been found scattered sparsely among the bases of the gonad epithelial cells from June to October. They were found in very much greater numbers in the May sample. Near the junction of the gonad with the mesenteric retractor muscle, these cells were present in very high concentrations and formed an almost continuous layer at the mesoglea/epithelium border. The presence of synaptinemal complexes in these cells strongly suggests that they are primary oocytes, and their small size and scant cytoplasm suggests that they are at a very early stage of differentiation.

Type II cells

These cells are similar in many respects to the Type I cells previously described, so only the points of difference between them will be discussed in detail. Type II cells are rather larger than Type I cells, reaching 15 μm in their largest dimension. This greater size is largely the result of an increase in the volume of cytoplasm, although the nucleus is slightly larger and may measure 6–9 μm in diameter.

Apart from size, the most obvious difference from Type I cells is the appearance of the nucleus. The nuclear material is of much greater overall electron density than that found in Type I cells, and is more uniform in density and appearance. The greater part of the nucleus contains a uniform finely granular material of moderate electron density. Areas of denser, more coarsely granular chromatin material are found scattered throughout the nucleus, and one or rarely two nucleoli are seen, always situated against the nuclear envelope (Figs. 9, 10 and 11).

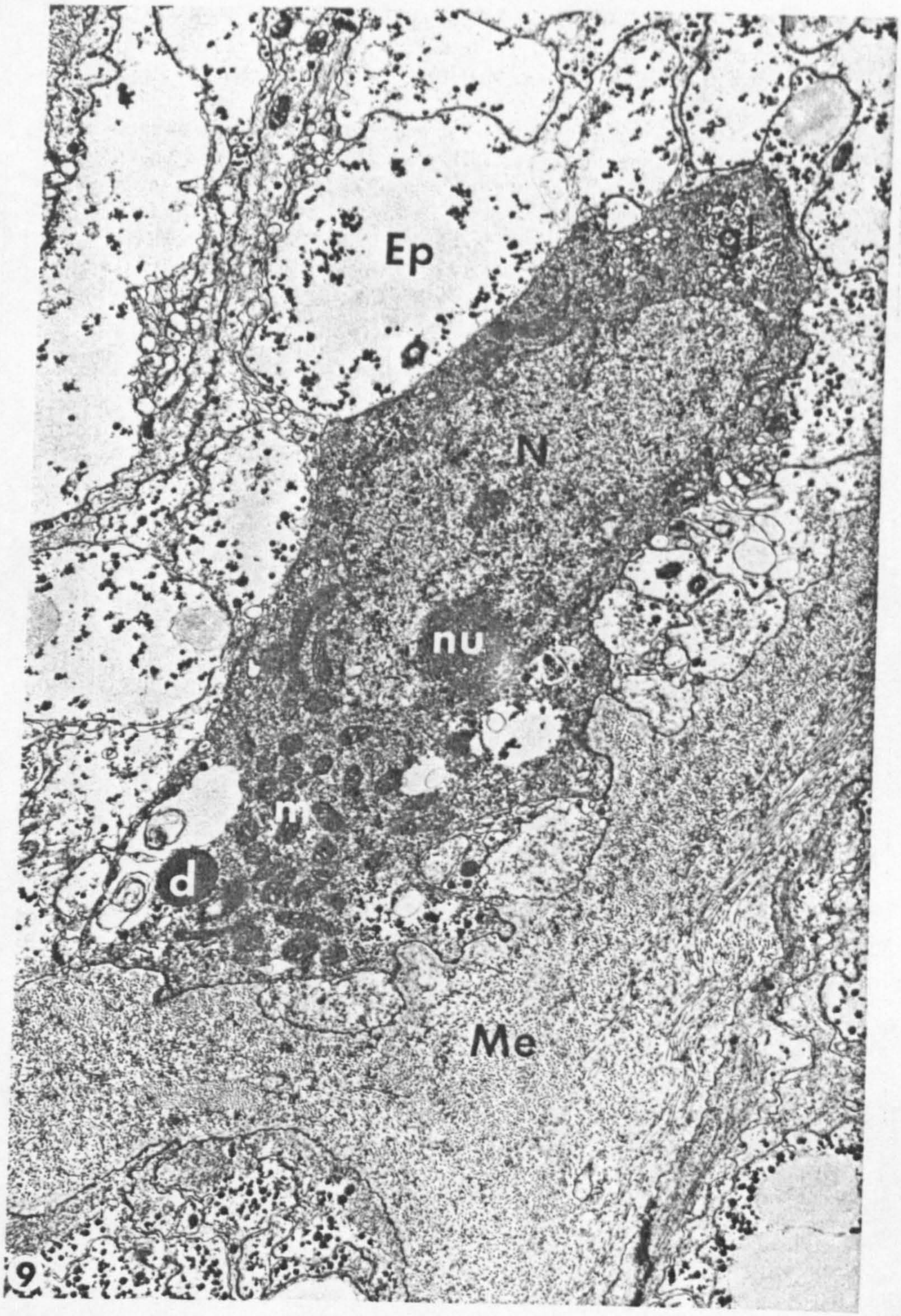
Dense chromatin granules, about 100 nm across, which are found occasionally in Type I cells, are found in all Type II cells, and may be present in considerable numbers (Fig. 11). The nucleus is bounded by a well-defined nuclear envelope which bears rather more nuclear pores than are found in Type I cells. Synaptinemal

Fig. 5. Part of a small Type I cell. The nucleus contains dense chromatin areas (ch), two of which form dense plaques against the nuclear envelope. Two vesicles containing numerous dense particles (arrowed) can also be seen. ×21000.

Fig. 6. Part of a Type I cell with an extensive Golgi complex (g) closely apposed to the nucleus (N). The cytoplasm also contains mitochondria (m), lipid droplets (L) and a lipid-like droplet surrounded by dense material (arrowed). ×16500.

Fig. 7. Part of a Type I cell, showing a group of mitochondria (m) close to the nucleus (N). Among the mitochondria is a honeycomb aggregation of dense material (asterisk). ×26000.

Fig. 8. Part of a Type I cell, outlined in ink for clarity. The cell contains mitochondria (m) and lipid droplets (L), and makes contact with the mesoglea (Me) by means of a slender cytoplasmic process extending between the endodermal muscle processes (mp). ×13500.



9

complexes are occasionally found in cells of this type, again as isolated short lengths, often making contact with the nuclear envelope (Fig. 10).

The cytoplasm is more extensive and more richly endowed with organelles and inclusions than in Type I cells. In particular the number of mitochondria and the amounts of glycogen and lipid are significantly increased. Type II cells also tend to be more variable in shape and irregular in outline than Type I cells.

The mitochondria are similar in appearance to those in Type I cells, and again tend to be arranged in one or more groups. The groups themselves however, may contain many more individual mitochondria, of which a higher proportion are elongate. Aggregations of dense material with the characteristic honeycomb appearance are again commonly found associated with the mitochondrial groups.

The glycogen is found localized into fewer, larger deposits within the cytoplasm, and more is found as larger rosettes of the α-conformation. Glycogen deposits are often associated with lipid droplets and dense bodies of various kinds.

Membrane-bound dense bodies (Figs. 9, 10 and 11) are more common than in Type I cells, and small amounts of endoplasmic reticulum may be found scattered randomly through the cytoplasm (Fig. 11).

Type II cells are found among the bases of the gonad epithelial cells at all times of the year, but especially between June and October. In June and July they were found in large numbers in the areas which in May had held high concentrations of Type I cells. The presence of synaptinemal complexes in the nuclei of Type II cells strongly suggests that they too are oocytes. Their larger size, greater cytoplasmic complexity and their periodicity in the gametogenic cycle suggests they are oocytes at a more advanced stage of differentiation than the Type I cells.

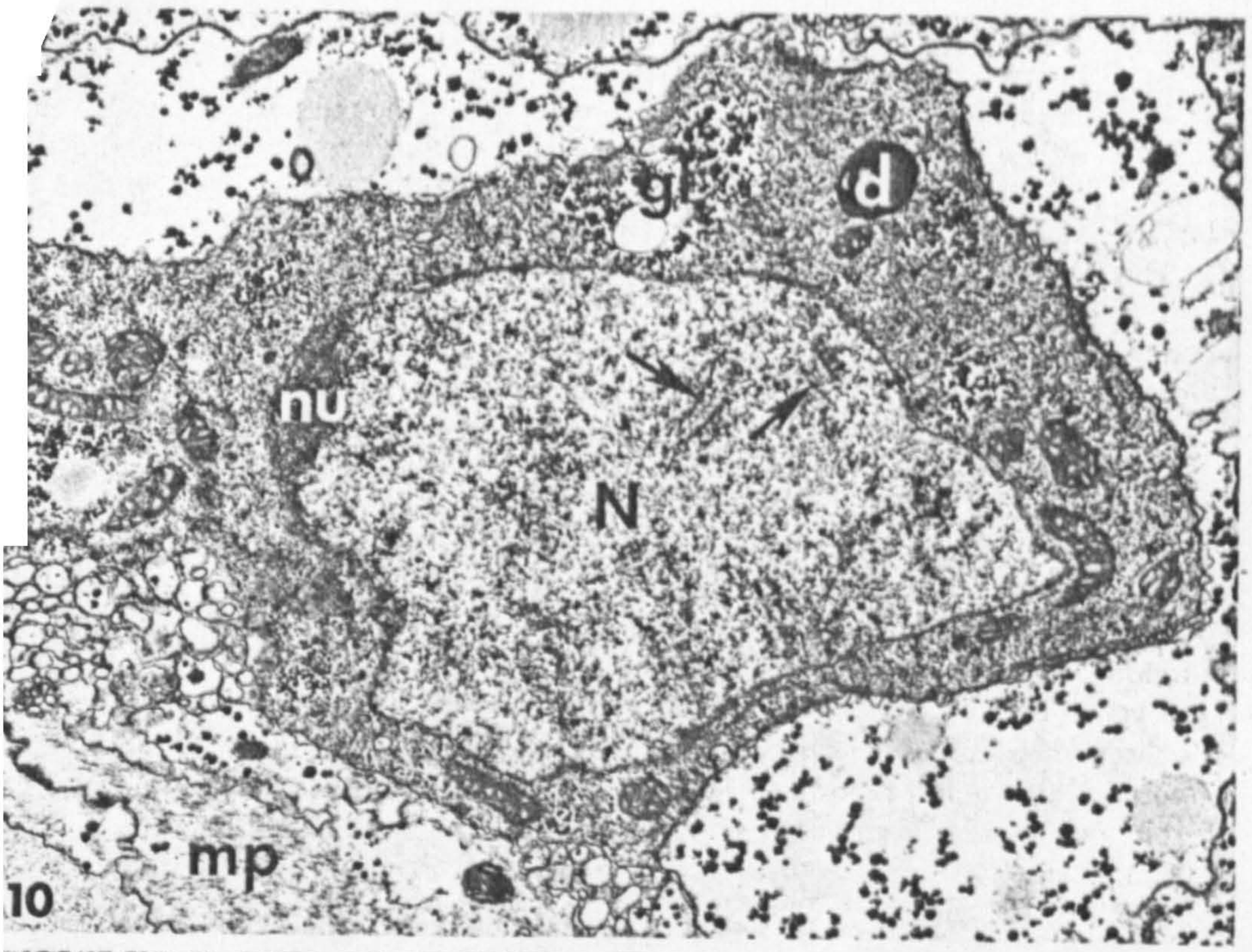
Type III cells

Type III cells are of similar size and appearance to Type II cells, averaging about 12 μm in greatest dimension. They are distinguished from Type II cells largely on the basis of their nuclear appearance. The Type III cell nucleus is large relative to the size of the cell, but is of generally low electron density. Irregular dense chromatin areas are separated by areas of very low electron density, in a manner reminiscent of the smaller Type I cells (Fig. 12). The nuclear envelope is always poorly defined, very often incomplete and sometimes apparently absent. Large seemingly empty vacuoles are often seen around the periphery of the nuclear area (Fig. 13).

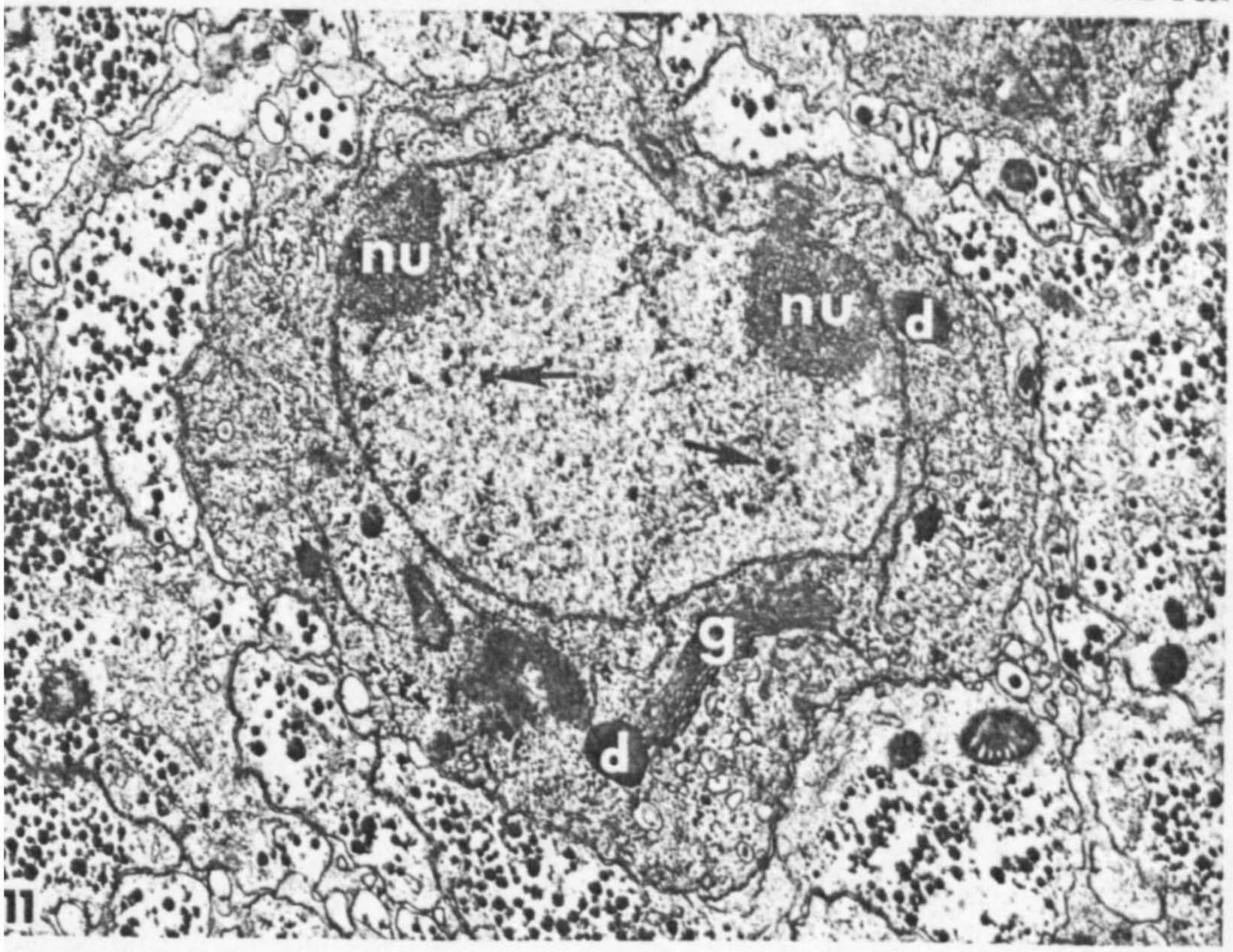
The cytoplasm may be quite extensive, and is similar in general appearance and organelle complement to that of Type II cells. Mitochondria may be numerous, but are usually small (Fig. 12). A flagellar basal-body–rootlet complex is sometimes found, but endoplasmic reticulum has not been seen in these cells.

Type III cells are again found among the bases of the gonad epithelial cells, but

Fig. 9. Type II cell, some 13 μm long, situated at the border between the mesoglea (Me) and the epithelial cell bases (Ep). The nucleus (N) is more dense than those of Type I cells, and contains a nucleolus (nu) and numerous dense chromatin granules. The cytoplasm contains an extensive group of mitochondria (m), glycogen deposits (gl) and a dense body (d). ×11500.



10



11

much less commonly than either of the preceding cell types. As yet they have only been found in the May sample, and then only in areas containing high concentrations of Type I cells.

Discussion

It has long been thought that sea anemone gametogenic cells arise in the endodermal epithelium of the mesenteries, and then migrate into the mesoglea where subsequent gamete maturation takes place [24, 25]. Recent studies of sea-anemone gametogenesis have confirmed this view. Dunn [7] describes how oocytes derive from small cells with relatively huge nuclei which arise and proliferate in the endoderm and migrate into the mesoglea when they have grown to a diameter of 20–25 μm. Similarly, Jennison [8] describes cells with large nuclei and little cytoplasm within the endoderm which grow and come to line the mesogleal/endodermal border before migrating into the mesoglea.

Actinia fragacea shows an annual cycle of gametogenic activity. This was first suggested by Carter and Funnell [22], and the study of which this paper forms a part confirms and extends their findings. Under the light microscope, small germ cells are common in the endoderm of the female gonad in June and July. They migrate into the mesoglea and most develop for a full year before being spawned or resorbed the following July. With the electron microscope, Type I cells were first seen in numbers in the endoderm in May, and had largely disappeared by July. Type II cells appeared in the endoderm in June, and were much less numerous by August. The temporal appearance and disappearance of these cells within the gonad is thus wholly consistent with the view that Type I cells represent an early, and Type II cells a slightly later stage in the differentiation of the female gametogenic cells. Their size and general appearance is also consistent with previously published descriptions of early female germ cells in other sea anemones.

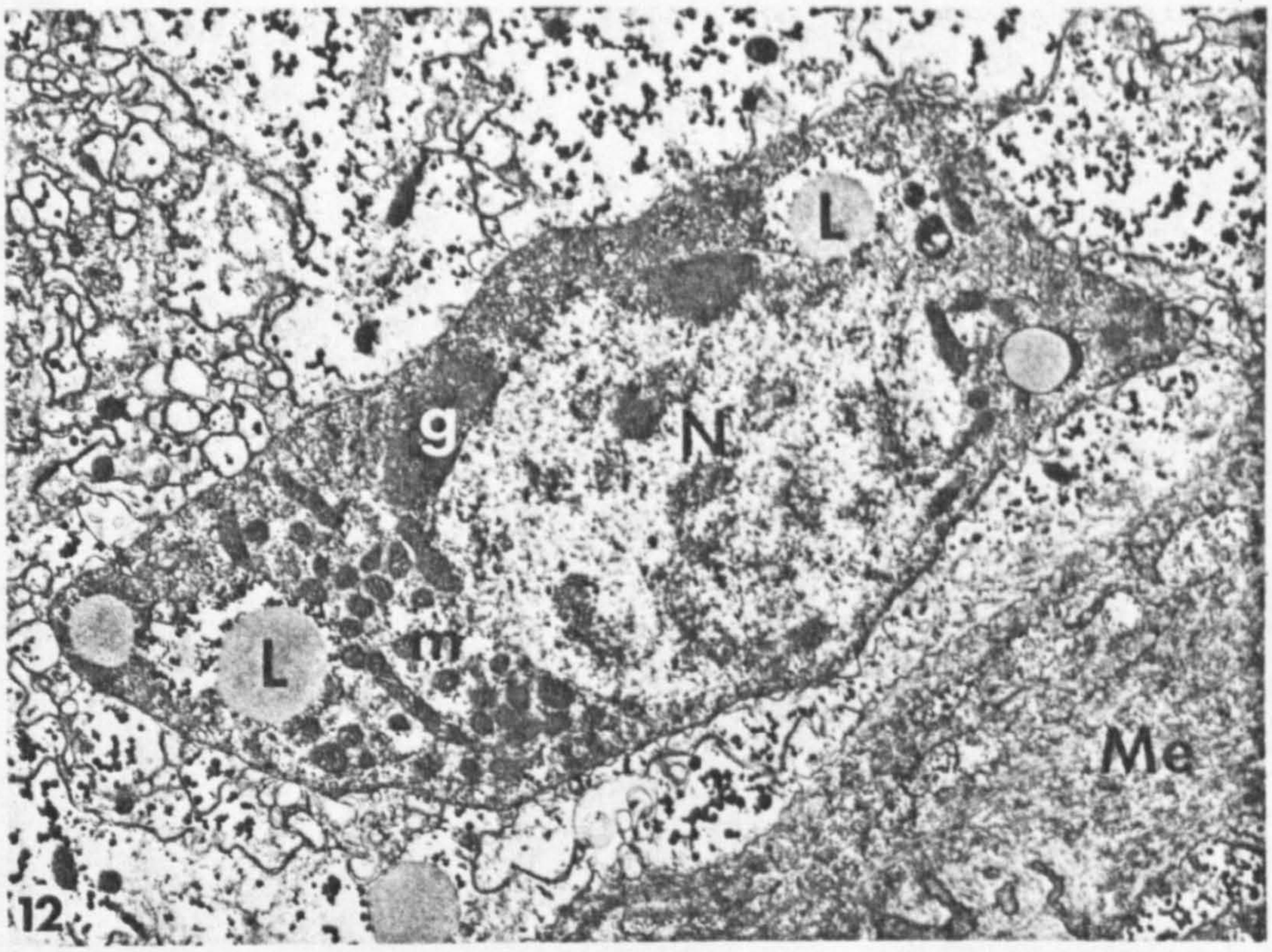
The view that Type I and Type II cells are early female germ cells is further supported by the finding of synaptinemal complexes within their nuclei. Synaptinemal complexes are characteristic of meiotic cells from many animal phyla [28] and have been found in anthozoan spermatocytes [2] including those of *A.fragacea* [23]. The presence of synaptinemal complexes is normally indicative of a cell at the pachytene or zygotene stage of meiotic prophase [28]. Their presence in Type I and II cells argues strongly that these cells are oocytes rather than oogonia or other possible precursor stages, or any other cell type.

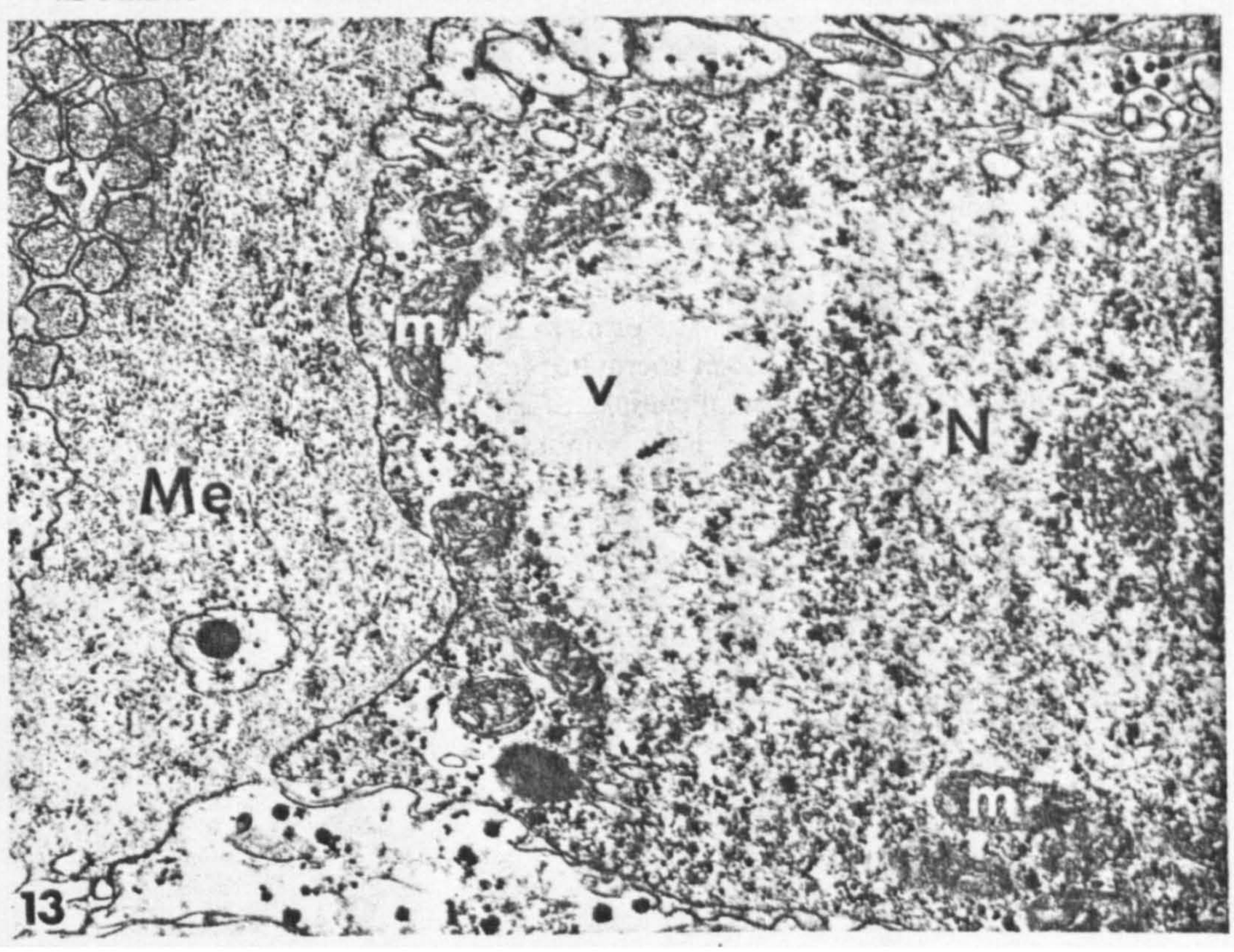
The suggestion that the female germ cells become oocytes while within the

---

Fig. 10. Type II cell. The nucleus (N) contains a nucleolus (nu) and synaptinemal complexes (arrowed). The cytoplasm contains several mitochondria, glycogen deposits (gl) and a dense body (d). ×13000.

Fig. 11. Type II cell. The nucleus contains two nucleoli (nu), both situated against the nuclear envelope, and numerous dense chromatin granules (arrowed). The cytoplasm contains a Golgi complex (g), dense bodies (d) and a few cisternae of endoplasmic reticulum (squat arrows). ×17000.


L
g
N
L
m
Me
12


cy
m
v
N
Me
m
13

endodermal cell layer, before entering the mesoglea, is at variance with some previously published information on sea anemone oogenesis. Schmidt and Schäfer [14] mention growing anthozoan oocytes still located in the endoderm before being surrounded by mesoglea. Both Dunn [7] and Jennison [8], however, describe cells 6–15 μm in diameter within the endoderm as primary oogonia. Larger cells within the endoderm, from 30–35 μm in *Epiactis* and from 15–25 μm and sometimes up to 45 μm in *Anthopleura*, are referred to as secondary oogonia. Both authors suggest that these cells do not cease mitotic cell division and become oocytes until they have entered the mesoglea. However, after extensive electron microscope observation, it appears that in *Actinia* there exists a coherent series of developing cell types, starting from Type I cells, leading to Type II cells and then larger oocytes which begin vitellogenesis and enter the mesoglea (unpublished observations), with no apparent evidence of cell division between the various stages.

Whilst the evidence very strongly points to Type I and Type II cells being early oocytes, the status of the Type III cells is less clear. Type III cells were only found in areas containing large numbers of very early oocytes. The possibility therefore arises that they might represent oogonia or some other proliferative stage in oocyte production. The clumped appearance of the chromatin in these cells and their lack of a well-defined nuclear envelope might be suggestive of cell division. However, no further evidence of mitosis was seen involving these cells, and it may well be that Type III cells represent a degenerate or arrested stage in oocyte development. Type III cells were only encountered rarely, and without further study little can be said about their significance.

There has been no previous detailed ultrastructural study of other anthozoan early oocytes with which those of *Actinia* could be usefully compared. However, from among the Hydrozoa, a number of early oocytes have been so described. In particular, Kessel [29] described all stages of oogenesis in some detail for an unidentified trachyline medusa, and Aizenshtadt [30] and Noda and Kanai [31, 32] have described the formation of early oocytes from interstitial cells in hydra. The early oocytes of *Actinia* seem to be generally similar in appearance to the corresponding cells in these hydrozoans. All show features characteristic of cells at a low level of differentiation—a large nucleus relative to the size of the cell, scant basophilic cytoplasm with numerous ribosomes but very little organized membrane systems. Indeed, the Type I cells of *Actinia* seem to have relatively even less cytoplasm than the other cell types, including the interstitial cells described by Noda and Kanai [31].

The arrangement of mitochondria in groups is common to all these types of cell.

Fig. 12. Type III cell, about 13 μm long, situated close to the mesoglea (Me). The nucleus (N) contains irregular dense chromatin areas, and the nuclear envelope is indistinct and incomplete. The cytoplasm contains a Golgi complex (g), lipid droplets (L) and numerous small mitochondria (m). ×9000.

Fig. 13. Part of a Type III cell, in contact with the mesoglea (Me). There is no obvious nuclear envelope present, and a large clear vacuole (v) is seen at the edge of the nuclear area (N). Cytospines (cy) of an adjacent large oocyte can be seen in the upper left-hand corner. ×23000.

The mitochondria in the *Actinia* oocytes are more compact and have a more electron dense matrix than those seen in hydra or described by Kessel. The honeycomb arrangement of dense, finely granular material associated with the groups of mitochondria in *Actinia* oocytes has not been described elsewhere, although both Aizenshtadt and Kessel report the presence of aggregations of finely filamentous or granular material in this location. Noda and Kanai [32] report the presence of 'germinal plasm' in many hydra cell types, most notably in interstitial cells, differentiating nematocytes and especially in oocytes, although this material is not closely associated with mitochondria. In those animal groups in which the germ plasm has been extensively studied, it usually consists of finely granular dense material closely associated with mitochondria. Whether similar material in *Actinia* oocytes can in any way be related to the germ plasm of hydra or other groups is of course uncertain.

Kessel also reports the presence in his early oocytes of basal-body–rootlet complexes and associated microtubules apparently similar to those described here for *Actinia*. Schmidt and Holtken [4], however, did not find such complexes in anthozoan oocytes. Kessel does not mention flagellar structures arising from these complexes. In *Actinia*, flagellar structures are commonly seen in very early oocytes. Rather larger oocytes (above 15 μm in diameter) are occasionally seen with complexes, but have never been seen with a flagellum (unpublished observations). It may be that the flagellum is lost early in oocyte development while the complex persists, at least for a time. In very early oocytes in *Actinia* the flagellum often arises at the bottom of a narrow invagination of the cell membrane. Thus the basal region of the flagellum is closely surrounded by the oocyte cytoplasm. This situation is strongly reminiscent of the 'cytoplasmic collar' found around the anterior portion of the tail flagellum of many anthozoan spermatozoa [6] including the sperm of *Actinia equina* [3]. The complex arrangement of microtubules in the basal region of the oocyte flagellum is also found in the sperm tail [3].

The cellular origin of the *Actinia* early oocytes remains unclear. In hydra and other hydrozoans, gametes are derived from undifferentiated interstitial cells which can also give rise to other cell types [24, 33]. In sea anemones, however, the role and occurrence of interstitial cells is a matter of some controversy. Robson [34] suggested that interstitial cells corresponding to those found in Hydrozoa did not occur in sea anemones. Van Praët [35] and Van Praët and Doumenc [36], investigating tentacle regeneration in *Actinia equina*, could not find interstitial cells with the light or electron microscope, and concluded that they were rare and played no part in wound healing or tentacle regeneration. Instead, regenerated tissues arose from dedifferentiated epithelial cells. Chapman [37] also felt that anthozoans did not possess interstitial cells as such, but that mesogleal cells and amoebocytes might fulfil a similar function. However, interstitial cells have been seen with the electron microscope by Singer [38] in regenerating *Aiptasia diaphana* and by Westfall [39] as the precursors of nematocytes in *Metridium senile*. Dunn [7] and Jennison [8] both presume that the gametes arise from interstitial cells, but with the light microscope can offer no evidence on this point.

The earliest oocytes in *Actinia* certainly resemble the interstitial cells of hydrozoans in ultrastructural appearance, but similar cells were not seen in the gonad at

other times of year. Perhaps interstitial cells are normally located elsewhere in the mesentery and migrate into the gonad during the phase of germ cell production. Whilst it is perhaps most likely that *Actinia* oocytes do arise from interstitial cells, on the present evidence other possible origins, such as from mesogleal cells or even by the dedifferentiation of other cell types cannot be ruled out.

The apparent absence of an obvious oogonial proliferative phase is of interest. No convincing signs of mitotic cell division were seen among any of the cell types described here. Type I cells appear in numbers in the endoderm of the gonad in May; a search of the April samples has so far not revealed the presence of any obvious precursor stages. This does not of course mean that such stages do not exist. Oocytes could arise from other cell types without a mitotic phase, or mitosis might take place extremely rapidly and so be seen only rarely in sectioned material, as has been suggested by Doumenc [40]. Unfortunately, the present study can shed no light on this point.

The finding of occasional very small oocytes at times of year well removed from the period of major oocyte differentiation in late Spring is as yet unexplained. A similar situation was described in *Anthopleura elegantissima* by Jennison [8]. It may be that oocytes continue to be produced at a low rate throughout much of the year, or that some oocytes produced in late Spring are retarded and develop only very slowly, possibly being spawned a season later than the majority.

Acknowledgements

The author is grateful to Dr. M.A. Carter for helpful discussion and critical reading of the manuscript, and to his Head of Department, Dr. F.A. Hibbert, for the provision of research facilities.

References

1 Lyke, E.B. and Robson, E.A., Spermatogenesis in Anthozoa; differentiation of the spermatid, Cell Tiss. Res., 157 (1975) 185–205.
2 Schmidt, M. and Zissler, D., Die Spermien der Anthozoen und ihre phylogenetische Bedeutung, Zoologica (Stuttg.), 44 (129) (1979) 1–98.
3 Larkman, A.U. and Carter, M.A., The spermatozoon of *Actinia equina* L. var. *mesembryanthemum*, J. Mar. Biol. Ass. U.K., 60 (1980) 193–204.
4 Schmidt, H. and Holtken, B., Peculiarities of spermatogenesis and sperm in Anthozoa. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, pp. 53–59.
5 West, D.L., Spermiogenesis in the anthozoan *Aiptasia pallida*, Tiss. Cell, 12 (1980) 243–253.
6 Hinsch, G.W., Comparative ultrastructure of cnidarian sperm, Am. Zool., 14 (1974) 457–465.
7 Dunn, D.F., Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill, 1869, Biol. Bull., 148 (1975) 199–218.
8 Jennison, B.L., Gametogenesis and reproductive cycles in the sea anemone *Anthopleura elegantissima* (Brandt, 1835), Can. J. Zool., 57 (1979) 403–411.
9 Rinkevich, B. and Loya, Y., The reproduction of the Red Sea coral *Stylophora pistillata*. I. Gonads and planulae, Mar. Ecol. Prog. Ser., 1 (1979) 133–144.

10 Rinkevich, B. and Loya, Y., The reproduction of the Red Sea coral *Stylophora pistillata*. II. Synchronization in breeding and seasonality of planulae shedding, Mar. Ecol. Prog. Ser., I (1979) 145–152.
11 Szmant-Froelich, A., Yevich, P. and Pilson, E.W., Gametogenesis and early development of the temperate coral *Astrangia danae* (Anthozoa: Scleractinia), Biol. Bull., 158 (1980) 257–269.
12 Spaulding, J.G., Embryonic and larval development in sea anemones (Anthozoa; Actiniaria), Am. Zool., 14 (1974) 511–520.
13 Dewel, W.C. and Clark, W.H., A fine structural investigation of surface specializations and the cortical reaction in eggs of the cnidarian *Bunodosoma cavernata*, J. Cell Biol., 60 (1974) 78–91.
14 Schmidt, H. and Schäfer, W.G., The anthozoan egg: trophic mechanisms and oocyte surfaces. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland, Biomedical Press, Amsterdam, 1980, pp. 41–46.
15 Schäfer, W.G. and Schmidt, H., The anthozoan egg: differentiation of internal oocyte structure. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, pp. 47–52.
16 Carter, M.A. and Thorpe, J.P., Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific, J. Mar. Biol. Ass. U.K., 61 (1981) 79–93.
17 Chia, F.S. and Rostron, M.A., Some aspects of the reproductive biology of *Actinia equina* (Cnidaria: Anthozoa), J. Mar. Biol. Ass. U.K., 50 (1970) 253–264.
18 Cain, A.J., Breeding system of a sessile animal, Nature, 247 (1974) 289–290.
19 Carter, M.A. and Thorp, C.H., The reproduction of *Actinia equina* L. var. *mesembryanthemum*, J. Mar. Biol. Ass. U.K., 59 (1979) 989–1001.
20 Gashout, S.E. and Ormond, R.F.G., Evidence for parthenogenetic reproduction in the sea anemone *Actinia equina*, J. Mar. Biol. Ass. U.K., 59 (1974) 975–987.
21 Stephenson, T.A., The British Sea Anemones, Vol. II, 426pp. Ray Society, London, 1935.
22 Carter, M.A. and Funnell, M.E., Reproduction and brooding in *Actinia*. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, pp. 17–22.
23 Larkman, A.U., Ultrastructural aspects of gametogenesis in *Actinia equina* L. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, pp. 61–66.
24 Campbell, R.D., Cnidaria. In A.C. Giese and J.S. Pearse (Eds.), Reproduction of Marine Invertebrates, Vol. I, Academic Press, New York, 1974, pp. 133–199.
25 Hyman, L.H., The Invertebrates: Protozoa through Ctenophora. The Acoelomate Bilateria, McGraw-Hill, New York, 1974.
26 Lyons, K.M., Collar cells in planula and adult ectoderm of the solitary coral *Balanophyllia regia* (Anthozoa, Eupsammiidae), Z. Zellforsch., 145 (1973) 57–74.
27 Vandermeulen, J.M., Studies on reef corals. II. Fine structure of the planktonic planula larva of *Pocillopora damicornis*, with emphasis on the aboral epidermis, Mar. Biol., 27 (1974) 239–249.
28 Westergaard, M. and von Wettstein, D., The synaptinemal complex, Annu. Rev. Genet., 6 (1972) 71–110.
29 Kessel, R.G., Electron microscope studies on developing oocytes of a coelenterate medusa with special reference to vitellogenesis, J. Morphol., 126 (1968) 211–248.
30 Aizenshtadt, T.B., Investigation of oogenesis in Hydra. I. Ultrastructure of interstitial cells at early stages of their transformation into oocytes, Sov. J. Dev. Biol., 5 (1975) 9–18.
31 Noda, K. and Kanai, C., An ultrastructural observation on *Pelmatohydra robusta* at sexual and asexual stages, with a special reference to 'germinal plasm', J. Ultrastruct. Res., 61 (1977) 284–294.
32 Noda, K. and Kanai, C., An ultrastructural observation on the embryogenesis of *Pelmatohydra robusta*, with special reference to 'germinal dense bodies'. In P. Tardent and R. Tardent (Eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press Amsterdam,, 1980, pp. 133–138.
33 Bode, H.R. and David, C.N., Regulation of a multipotent stem cell, the interstitial cell of hydra, Prog. Biophys. Mol. Biol., 33 (1978) 189–206.

34 Robson, E.A., The structure and hydromechanics of the musculoepithelium in *Metridium*, Q. J. Microsc. Sci., 98 (1957) 265–278.
35 Van Praët, M., Règénération de la région péri-orale d'*Actinia equina*, Thèse de l'Université de Paris, 1974.
36 Van Praët, M. and Doumenc, D., Morphologie et morphogènèse expérimentale du tentacule chez *Actinia equina*, J. Microsc. Biol. Cell., 23 (1975) 29–38.
37 Chapman, D.M., Cnidarian histology. In L. Muscatine and H.M. Lenhoff (Eds.), Coelenterate Biology. Reviews and New Perspectives, Academic Press, New York, 1974, pp. 2–92.
38 Singer, I.I., Tentacular and oral disc regeneration in the sea anemone *Aiptasia diaphana*. III. Autoradiographic analysis of patterns of tritiated thymidine uptake, J. Embryol. Exp. Morphol., 26 (1971) 253–270.
39 Westfall, J.A., The differentiation of nematocysts and associated structures in the Cnidaria, Z. Zellforsch., 75 (1966) 381–403.
40 Doumenc, D.A., Etude dynamique de la morphogènèse au course des phases Actinella et Edwardsia de l'actinie *Cereus pedunculatus* Pennant, Arch. Zool. Exp. Gén., 118 (1977) 79–102.

International Journal of Invertebrate Reproduction, 4 (1982) 375–379
Elsevier Biomedical Press

Short communication

Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a nutritive function

A.U. Larkman and M.A. Carter

Department of Biological Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth, U.K.

(Received 17 August 1981)

The developing oocyte of the sea anemone *Actinia fragacea* is associated with a distinct group of gonad epithelial cells which constitute the trophonema. Electron microscopy has shown that the cells of the trophonema extend through a pore in the mesoglea which surrounds the oocyte, and make intimate contact with the oocyte surface. The ooplasm beneath this region of contact differs from the rest of the oocyte in containing numerous small vesicles, but few yolk granules or other organelles. Light microscope autoradiography has shown that oocytes within gonads can take up and incorporate tritiated glucose and leucine from solution. The cells of the trophonema appear more active in precursor incorporation than other gonad epithelial cells. The evidence therefore suggests that these cells have a nutritive function during oogenesis.

trophonema; oogenesis; *Actinia fragacea*; ultrastructure; autoradiography

Introduction

The gonads of sea anemones are found on the mesenteries, between the filaments and the retractor muscles [1]. Like the rest of the mesentery, the gonads consist of two layers of endodermal cells separated by the mesoglea. Oocytes originate in the endoderm but penetrate and come to lie entirely within the mesoglea at an early stage in their development. It is in the mesoglea that most oocyte growth and vitellogenesis occurs.

A notable feature of the female gonad of several sea anemones is the presence of distinct groups of endoderm cells which can be seen to be associated with many of the oocytes. This structure is called the trophonema; it was first described by Hertwig and Hertwig [2] and was later discussed by Nyholm [3]. Recently, it has been observed in *Epiactis prolifera* by Dunn [4], *Anthopleura elegantissima* by Jennison [5] and in several anthozoan species by Schmidt and Schäfer [6]. Although there may be some variation between species, several authors have suggested that the trophonema has a nutritive function [2–4,6].

We have used the techniques of electron microscopy and light microscope

0165-1269/82/0000–0000/$02.75

autoradiography in an attempt to determine the function of the trophonema in *Actinia fragacea*.

Electron microscopy

Anemone gonads were sampled regularly throughout the year and gonad material was prepared for electron microscopy by standard techniques as described previously [7]. Thus we were able to observe oocytes at all stages of development. For the purpose of this study we concentrated on oocytes between 60 and 120 μm in diameter which were thought to be active in growth and vitellogenesis.

The major electron microscope observations are summarized in Fig. 1. The trophonema is outlined by a depression in the surface of the endoderm. The underlying cells are slender and are held together by prominent intercellular junctions. The cells extend through a pore in the mesoglea and terminate in a shallow depression in the surface of the oocyte. The mesoglea is thickened locally to form a lip around the pore. The trophonema is the only site where the oocyte is in direct contact with endodermal cells; elsewhere they are separated by a usually thin layer of mesoglea. The area of cellular contact under the trophonema is increased by the projection of oocyte cytospines into indentations in the bases of the trophonemal cells.

As has been described by previous authors [4–6], the germinal vesicle is displaced towards the trophonema, leaving only a narrow band of ooplasm in this region,

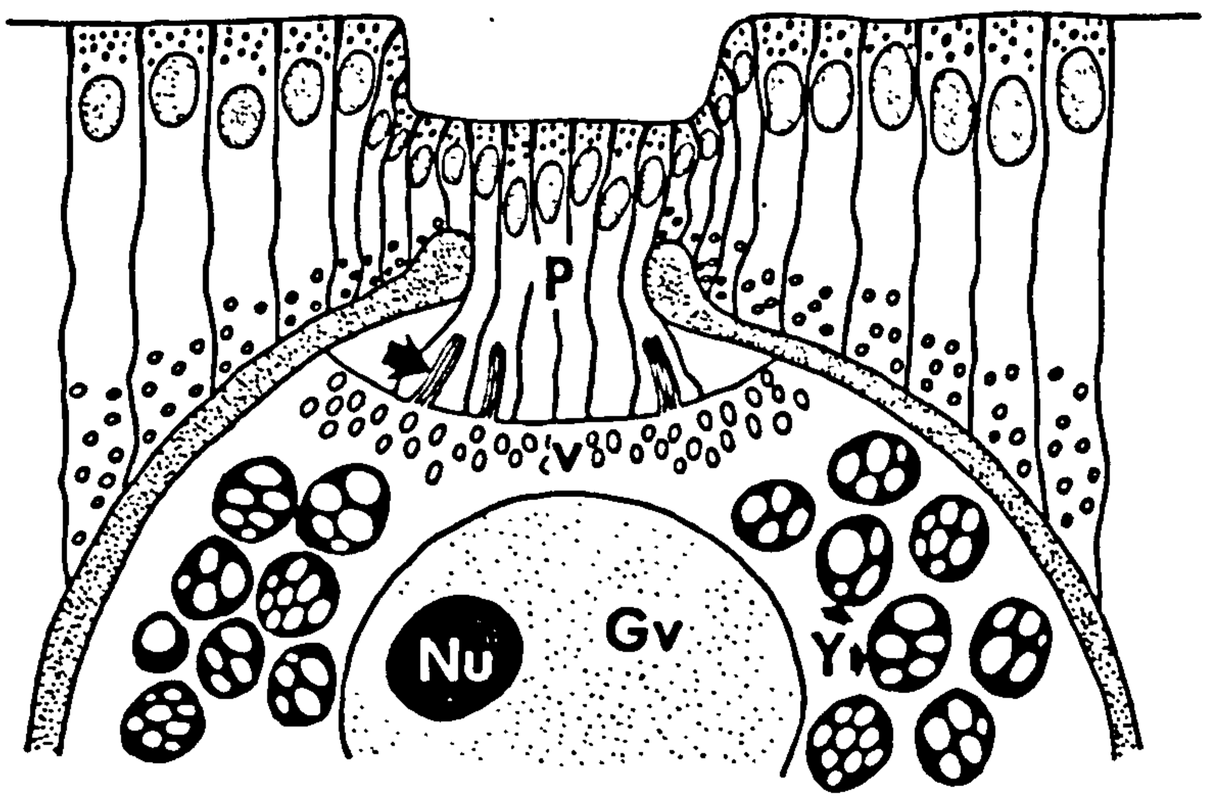


Fig. 1. Simplified diagram showing major morphological features of the trophonema. Trophonemal cells extend through a pore (P) in the mesoglea (stippled) surrounding the oocyte and make contact with the oocyte surface. Cytospines (arrow) project from the oocyte surface into indentations in the bases of the trophonemal cells. The oocyte germinal vesicle (Gv) with its single large nucleolus (Nu) is displaced towards the trophonema. The band of ooplasm beneath the trophonema contains numerous small vesicles (v) but few yolk granules (Y).

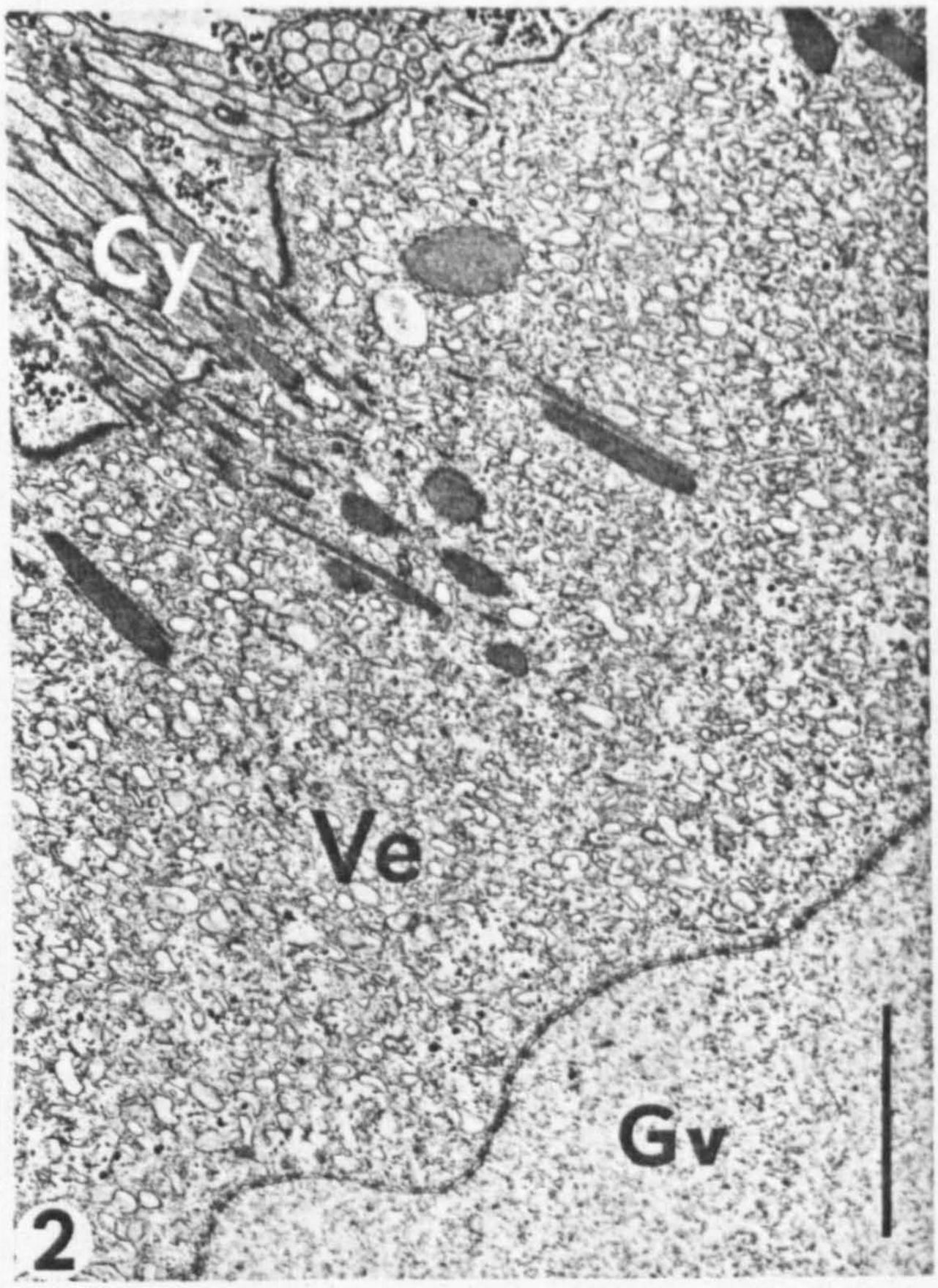


Fig. 2. Electron micrograph of ooplasm between the trophonema and the germinal vesicle (Gv) showing the presence of numerous small vesicles (Ve). Groups of cytospines (Cy) can be seen projecting from the oocyte surface into the bases of the trophonemal cells. Scale line = 2 μm.

which we have found to differ from the rest of the oocyte. It contains large numbers of small, membrane bound vesicles, 200–500 nm in diameter (Fig. 2).

Autoradiography

The uptake of tritiated glucose and leucine from solution was examined autoradiographically. Pieces of female gonad containing developing oocytes can be excised and maintained in sterile culture for some weeks. It was therefore possible to incubate gonad pieces in vitro. Radiolabelled chemicals were dissolved in sterile sea

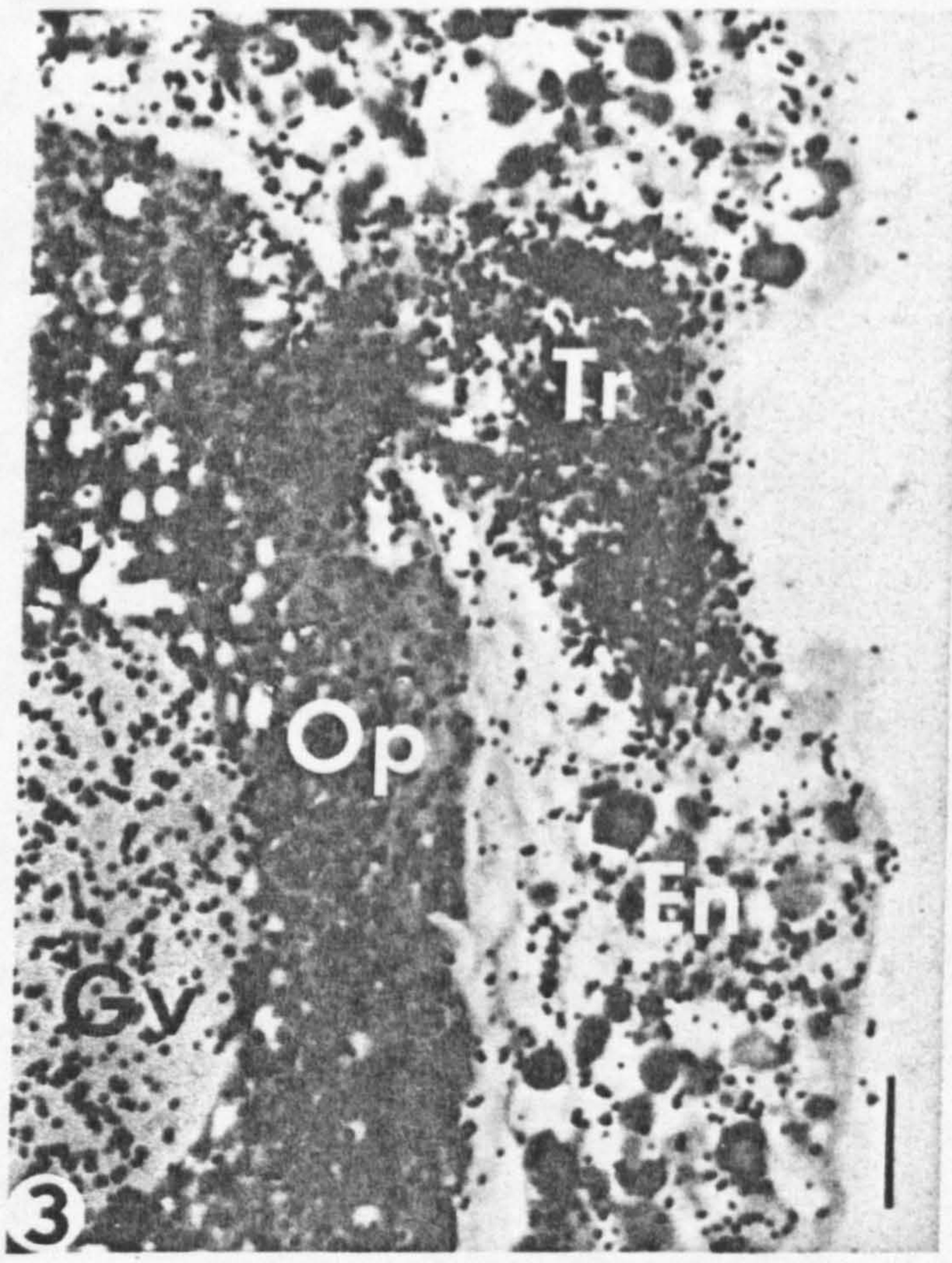


Fig. 3. Light microscope autoradiograph of area of gonad after incubation in tritiated leucine. The oocyte is heavily labelled, both over the ooplasm (Op) and the germinal vesicle (Gv). The trophonema (Tr) is much more heavily labelled than the surrounding endoderm (En). Scale line = 10 μm.

water at 10 μCi/ml, and incubation times varied between 1 and 24 h. Unlabelled chase times ranged between 1 and 72 h. Gonads were fixed in glutaraldehyde, embedded in JB4 plastic resin and sectioned at 2 μm. The sections were dip-coated with Ilford K2 liquid emulsion and exposed at 4°C for 5–8 days.

With the procedure outlined above, developed silver grains generally indicate sites where the labelled precursors have been incorporated into macromolecules. Full details of the method and the controls used will be described in a future publication.

After incubation in tritiated glucose, heavy concentrations of silver grains were found over the trophonema and over regions of the oocyte which electron microscope studies had shown were rich in glycogen. Other endodermal cells were

labelled, especially over their basal regions, but much more lightly than the trophonema. With long chase times, more label was found over the oocyte and less over the trophonema.

After incubation in tritiated leucine, the trophonema was again heavily labelled in comparison with the rest of the endoderm (Fig. 3). The oocyte was labelled generally, but the germinal vesicle and in particular the nucleolus were heavily labelled. Long chase times did not appear to affect the distribution of silver grains significantly.

The endodermal cells presumably convey nutrients from the enteron fluid to the growing oocytes. The electron microscope studies have shown that trophonemata provide regions of intimate contact between endoderm and oocytes. Autoradiography has shown that oocytes (within gonads) can incorporate small molecules from solution and that trophonemata appear much more active in incorporation than the rest of the endoderm. This evidence points to a nutritive function for the trophonema during oocyte growth. We have observed a structure resembling the trophonema in the testicular cyst of the male gonad which gives similar autoradiographic results.

References

1 Hyman, L.H., The Invertebrates: Protozoa through Ctenophora, McGraw-Hill Book Co., New York and London, 1940, 726 pp.

2 Hertwig, O. and Hertwig, R., Die Actinien, Verlag von Gustav Fischer, Vornals Friedr. Mauke, Jena, 1879, 224 pp.

3 Nyholm, K.G., Zur Entwicklung und Entwicklungsbiologie der Ceriantharien und Aktinien, Zool. Bidr. Uppsala, 22 (1943) 87–248.

4 Dunn, D.F., Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill 1869, Biol. Bull. (Woods Hole, Mass.), 148 (1975) 199–218.

5 Jennison, B.L., Gametogenesis and reproductive cycles in the sea anemone *Anthopleura elegantissima* (Brandt, 1835), Can. J. Zool., 57 (1979) 403–411.

6 Schmidt, H. and Schäfer, W.G., The anthozoan egg: trophic mechanisms and oocyte surfaces. In P. Tardent and R. Tardent (eds.), Developmental and Cellular Biology of Coelenterates, Elsevier/North-Holland Biomedical Press, Amsterdam, 1980, pp. 41–46.

7 Larkman, A.U., An ultrastructural study of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria: Anthozoa), Int. J. Invertebr. Reprod., 4 (1981) 147–167.

JOURNAL OF MORPHOLOGY 178:155–177 (1983)

An Ultrastructural Study of Oocyte Growth Within the Endoderm and Entry Into the Mesoglea in *Actinia fragacea* (Cnidaria, Anthozoa)

ALAN U. LARKMAN
Department of Biological Sciences, Portsmouth Polytechnic, Portsmouth, PO1 2DY, United Kingdom

ABSTRACT Sea anemone gametes arise in the endoderm but migrate into the mesoglea at an early stage. In order to observe this process, large individuals of *Actinia fragacea* were collected from the same intertidal location at regular intervals over a 2-year period, and their gonads were examined by light and electron microscopy.

The cellular origin of the oocytes is unclear, but the smallest recognizable oocytes are rounded cells, 6–8 μm in diameter, with relatively large nuclei which may contain synaptinemal complexes. Their cytoplasm contains numerous ribosomes, a flagellar basal-body-rootlet complex, and distinctive dense structures also present in male germ cells but not found in anemone nongerminal cells. During the endodermal phase of growth, the density of the oocyte nucleus increases, a single nucleolus becomes prominent, and mitochondria and glycogen accumulate in the cytoplasm. Most oocytes, but not all, only begin major vitellogenesis after entry into the mesoglea. Most oocytes enter the mesoglea before they attain a diameter of 25 μm.

The oocytes migrate toward and enter the mesoglea by a process resembling amoeboid movement. During entry, the oocytes are constricted into a characteristic "hourglass" shape and become covered by a basal lamina continuous with that of the gonad epithelium. The last part of the oocyte to enter the mesoglea forms an intimate relationship with the surrounding endodermal cells, which is maintained after entry is complete, and is thought to be important in the establishment of the trophonema.

The gonads of sea anemones are located on the mesenteries and lie between the mesenteric retractor muscles and the gastric filaments (Campbell, '74). Like the rest of the mesentery, the gonads consist of two layers of endodermal epithelial cells separated by a layer of mesoglea. The germ cells arise among the endodermal epithelial cells, but at an early stage in their development they migrate into the mesoglea (Hyman, '40; Campbell, '74). Thus the major part of growth and differentiation of the gametes takes place while they are surrounded by a layer of fibrous, sparsely cellular mesogleal material. This paper describes fine structural aspects of the early stages of oogenesis in the sea anemone *Actinia fragacea* and covers the period of oocyte growth within the endoderm and the subsequent entry of the oocytes into the mesoglea.

Our knowledge of gametogenesis in sea anemones is as yet only fragmentary. However, an understanding of the cellular transformations involved in gametogenesis in these animals is of interest partly because of their ecological significance and also because such studies in these primitive metazoans may give a perspective to studies of corresponding processes in higher organisms. Light microscope descriptions of all stages of gametogenesis have recently been published for the anemones *Epiactis prolifera* (Dunn, '75) and *Anthopleura elegantissima* (Jennison, '79). Less complete data are available for *Actinia equina* (Chia and Rostron, '70; Carter and Funnell, '80), *Peachia quinque-*

capitata (Spaulding, '72), *Tealia crassicornis* (Chia and Spaulding, '72), and the anthozoans *Ptilosarcus guerneyi* (Chia and Crawford, '73), *Stylophora pistillata* (Rinkevich and Loya, '79), and *Astrangia danae* (Szmant-Froelich et al., '80). The electron microscope has been less extensively used, and recent reviewers have commented on the paucity of ultrastructural information available on anthozoan gametogenesis (Chapman, '74; Roosen-Runge, '77; Nieuwkoop and Satasurya, '81). The pioneering work of Spaulding ('74), Dewel and Clark ('74), and Clark and Dewel ('74) has not been greatly extended. Studies of anthozoan oogenesis in particular have been scarce (Schmidt and Schäfer, '80; Schäfer and Schmidt, '80; Larkman, '80). There has been no ultrastructural account of the entire process of spermatogenesis or oogenesis for any single anemone species, and the early stages of gametogenesis, which may be difficult to visualize with the light microscope, are still poorly understood. An electron microscope study of gametogenesis in the sea anemone *Actinia fragacea* was therefore undertaken. In order that all stages of the process might be observed, anemones were collected from the field at regular intervals throughout the year, and their gonads were examined by light and electron microscopy. A previous paper (Larkman, '81) discussed the occurrence and identification of early female germ cells within the endoderm.

Actinia fragacea is an intertidal sea anemone found on rocky shores around the southwestern coast of England and elsewhere. Until recently it was considered to be a variety of the widely distributed beadlet anemone *Actinia equina*, but it is now thought to be a separate species (Rostron and Rostron, '78; Carter and Thorpe, '81). Unlike *A. equina* it does not brood its young within the gastrovascular cavity, and it is thought to be oviparous (Stephenson, '35).

MATERIALS AND METHODS

Samples of five or six large individuals of the sea anemone *Actinia fragacea* were collected at approximately monthly intervals from a rocky shore at Wembury, near Plymouth, England, over a 2-year period extending from 1979 to 1981. Gonads were removed by dissection and fixed by immersion in 3% glutaraldehyde in a 0.1 M phosphate buffer to which 3% sodium chloride had been added. Material for electron microscopy was postfixed in 1% osmium tetroxide in the same buffer, dehydrated using ethanol, and embedded in "EMix" epoxy resin (EMscope Laboratories Ltd.). Ultrathin sections were cut on an LKB Ultratome III using glass or diamond knives, stained with uranyl acetate and lead citrate, and examined using a Philips EM300 electron microscope.

Material for light microscopy was embedded in JB4 plastic resin (Polysciences Inc.) and sectioned at 1–2 μm on an LKB Pyramitome using glass knives. Sections were routinely stained with 1% toluidine blue in 1% sodium tetraborate solution.

RESULTS

Growth within the endoderm

The oocytes of *Actinia fragacea* arise in the endoderm of the mesenteries, where they remain and grow for a variable period before entering the mesoglea. Very small oocytes

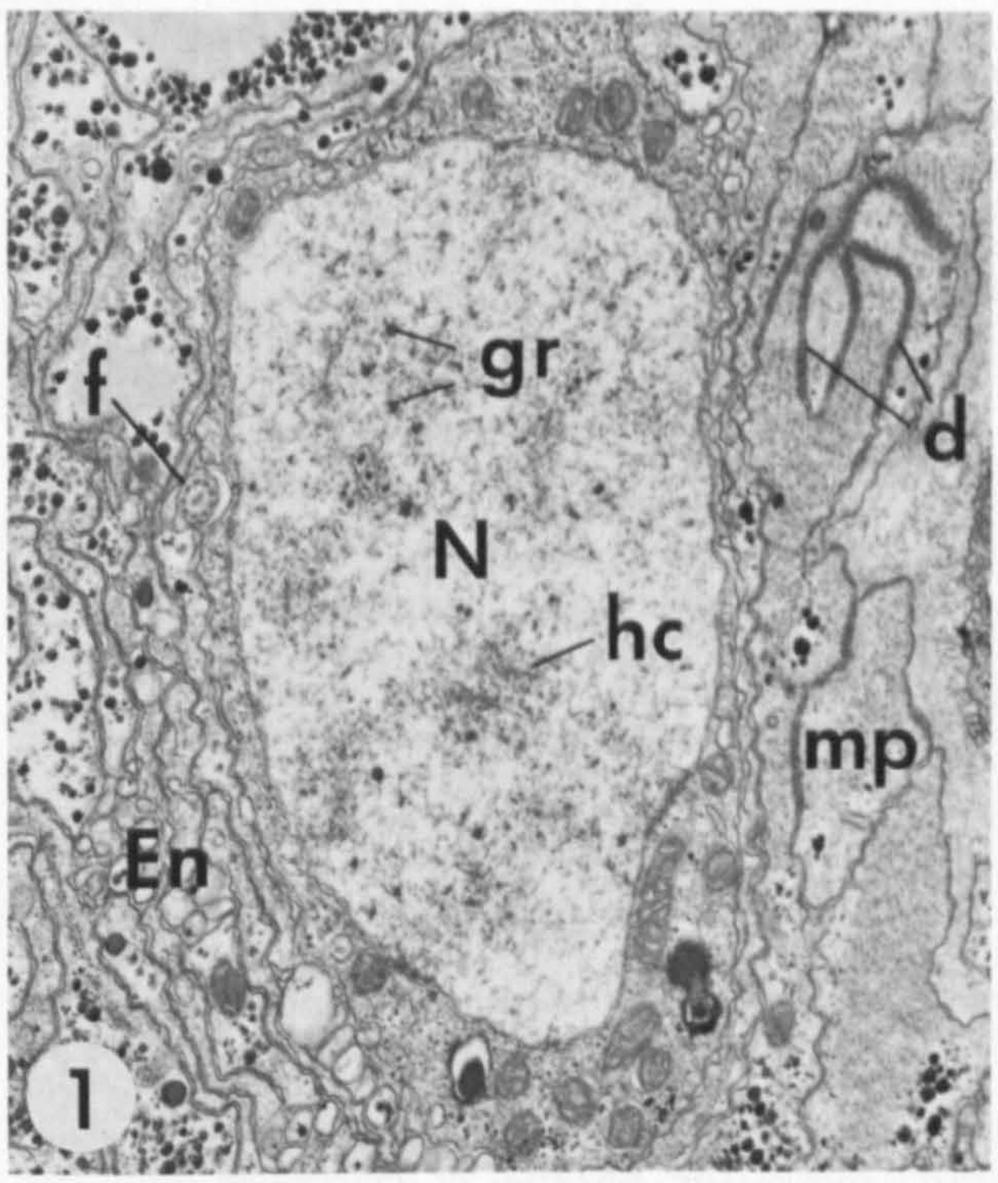
Fig. 1. A small oocyte, some 6.5 μm from top to bottom, lying in the endoderm close to the muscle processes and the mesoglea. ×14,000.

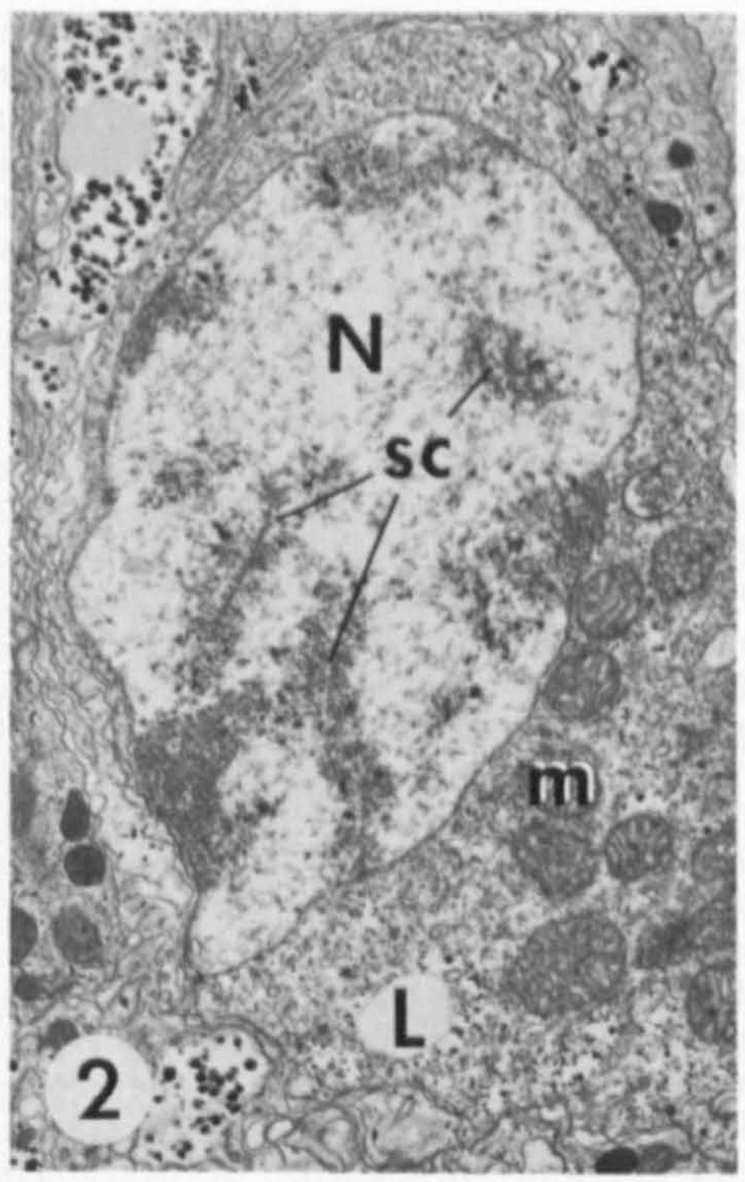
Fig. 2. A small endodermal oocyte with synaptinemal complexes. ×11,000.

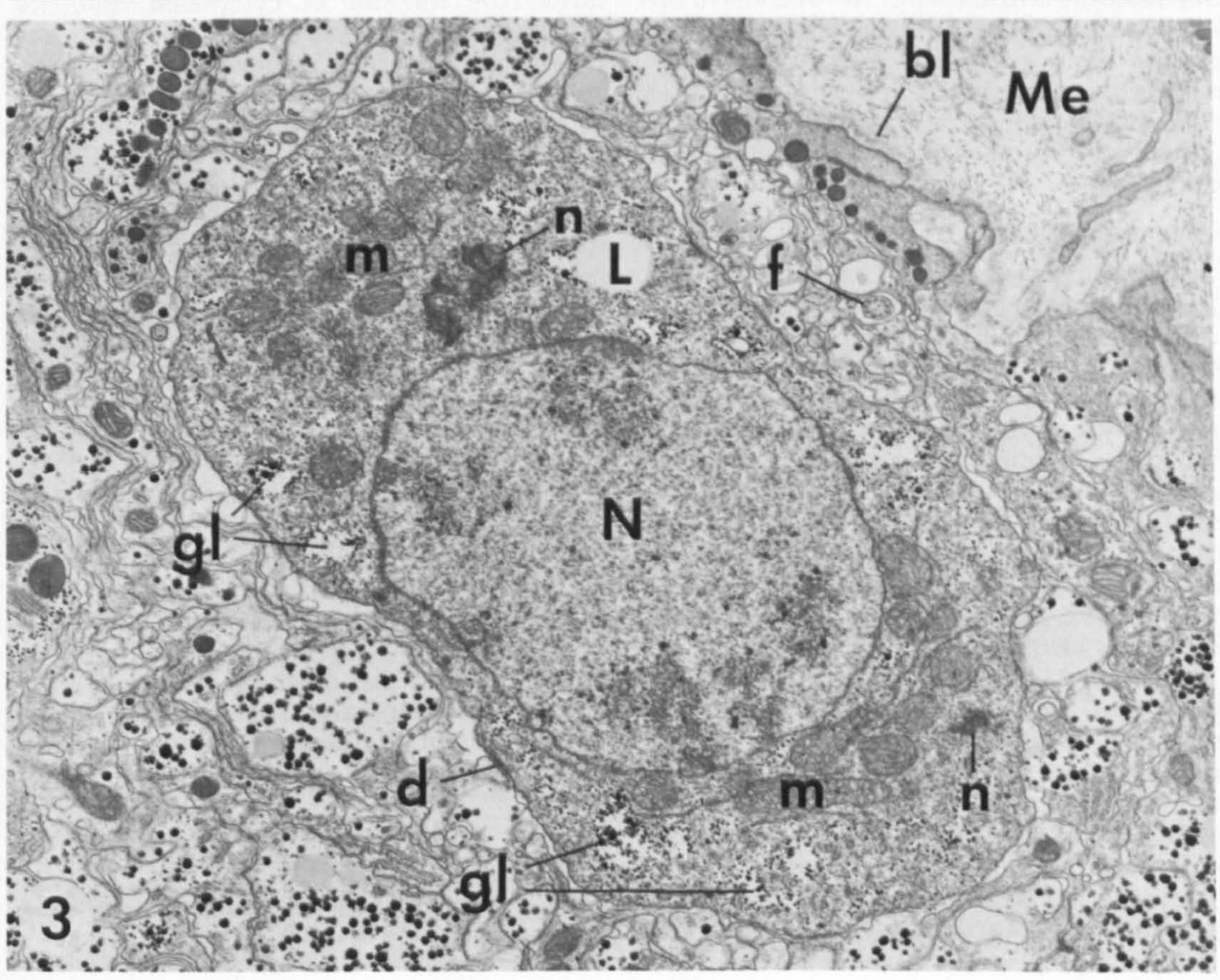
Fig. 3. An endodermal oocyte some 9.5 μm in length lying close to the mesoglea. Note the increase in nuclear density compared with Figures 1 and 2. The nearby flagellum probably arises from the oocyte. ×12,500.

Abbreviations

Ac, Amoebocyte
bl, Basal lamina
c, Centriole
cv, Coated vesicles
cy, Cytospines
d, Desmosome
db, Dense body
dc, Area of dense cytoplasm
En, Endoderm
EO, Endodermal portion of oocyte
er, Endoplasmic reticulum
f, Flagellum
fb, Nuclear fibrillar body
fy, Forming yolk body
g, Golgi complex
gc, Granular core of nuclear fibrillar body
gl, Glycogen
gr, Nuclear granule
hc, Heterochromatin
L, Lipid droplet
m, Mitochondrion
Me, Mesoglea
MO, Mesogleal portion of oocyte
mp, Endodermal muscle process
mv, Multivesicular body
N, Nucleus
n, Possible nuage material
nd, Dense component of nuage
ne, Nuclear envelope
nf, Fibrillar component of nuage
nu, Nucleolus
Oc, Oocyte
op, Oocyte process
sc, Synaptinemal complex
sr, Striated rootlet
tu, Aggregation of tubules
v, Vesicles
va, Vacuole
y, Yolk body


f
gr
d
N
hc
mp
En
1


N
sc
m
L
2


bl
Me
n
m
L
f
N
gl
d
m
n
gl
3

were first recognized in the endoderm in late spring, when they may occur in large numbers. They are small cells, 6–8 μm in diameter, with relatively large nuclei and dense cytoplasm. The occurrence and identification of these cells has been described in a previous paper (Larkman, '81). The number of these cells present in the endoderm decreases through the summer until by autumn virtually all have entered the mesoglea.

While within the endoderm, the oocytes are surrounded by the fine basal processes of the endodermal epithelial cells. Some oocytes lie actually in contact with the mesoglea, but most are situated a short distance from it (Fig. 1). The smallest oocytes are roughly spherical, while slightly larger ones (8–12 μm diameter) are often elongate. Oocytes above 12 μm are usually spherical once more. Oocytes within the same gonad do not develop in synchrony, and even oocytes of the same size may vary in their apparent degree of differentiation. The size at which the oocytes enter the mesoglea also varies considerably. The major features of the oocyte nucleus and cytoplasm and the changes which they undergo while within the endoderm are described below. Some oocytes begin vitellogenesis while within the endoderm, though most begin only after entry into the mesoglea. Vitellogenesis will be the subject of a separate paper, and so will not be described here.

Nuclear changes

In very small oocytes, up to about 10 μm in diameter, the nucleus is characteristically of very low electron density (Fig. 1). It contains irregular areas of more dense chromatin material which often contact the nuclear envelope. Small dense granules, 50–80 nm in diameter, are found scattered throughout the nucleus. A nucleolus is often seen, but it is usually poorly defined and closely associated with the other dense nuclear material. Tripartite synaptinemal complexes are often found in the nuclei of these small oocytes (Fig. 2; see Larkman, '81).

As the oocyte enlarges, the overall electron density of the nucleus progressively increases (Figs. 1–6), until in oocytes over 15 μm the nucleus may appear more dense than the surrounding cytoplasm (Fig. 7). The patches of heterochromatin become less obvious, and dense fibrillar bodies appear within the nucleus (Fig. 7). These consist of aggregations of fibrillar material, often surrounding a finely granular core (Fig. 8). They may reach 1–2 μm in diameter, and up to five such bodies have been seen in a single section of a nucleus. The nucleolus becomes larger and more clearly defined, and is spherical, finely granular, and homogenous (Figs. 6, 7). It may reach 8-μm diameter in a 35-μm oocyte. The nucleolus is usually located to one side of the nucleus and is often surrounded by dense nuclear granules of the type described above (Fig. 9). Synaptinemal complexes have not been observed in oocytes above 12 μm in diameter.

Throughout oocyte growth, the nucleus is bounded by an intact, typical, double membrane nuclear envelope, bearing nuclear pores. The pores become more numerous and more closely packed as the nucleus enlarges.

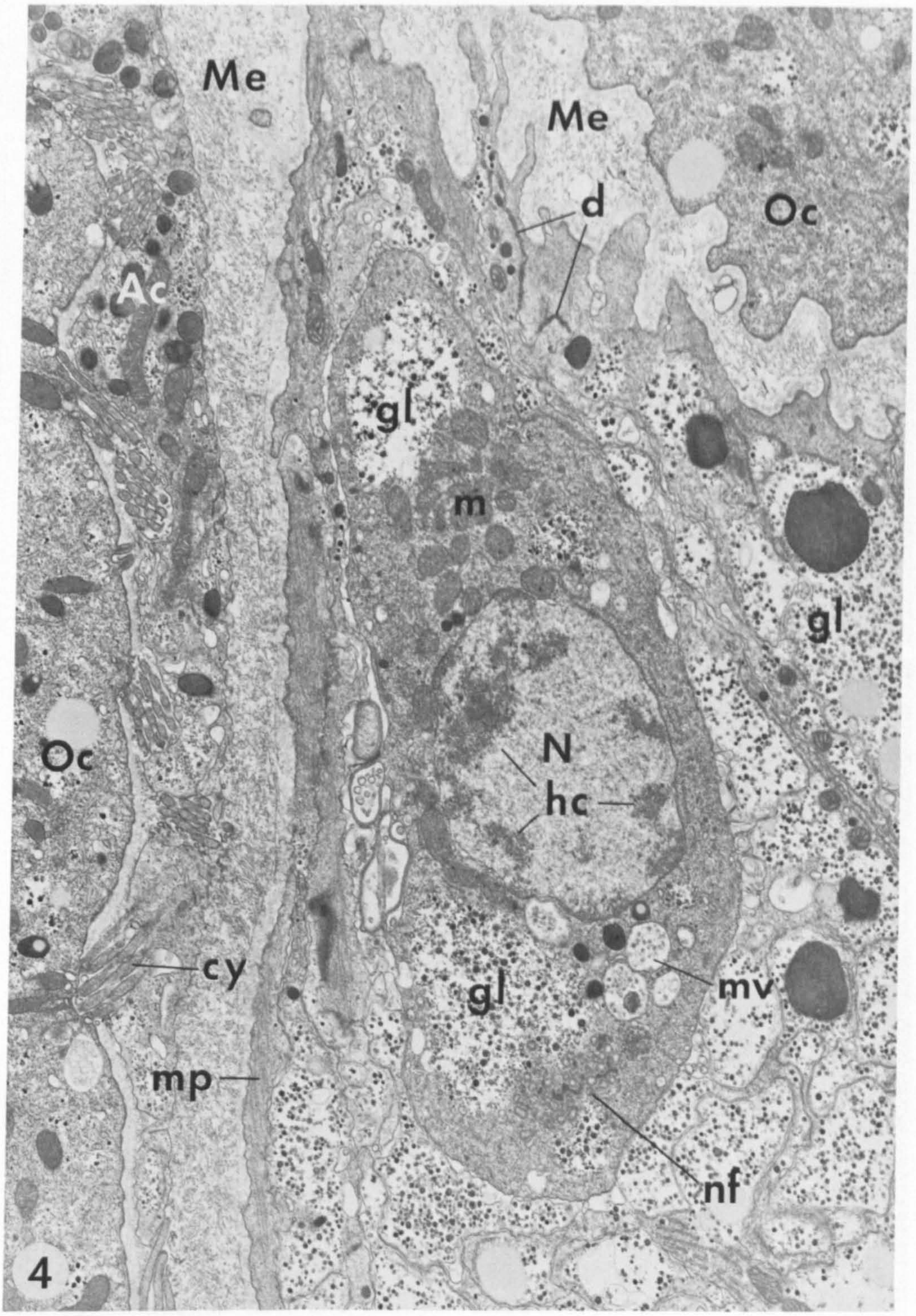
Cytoplasmic changes

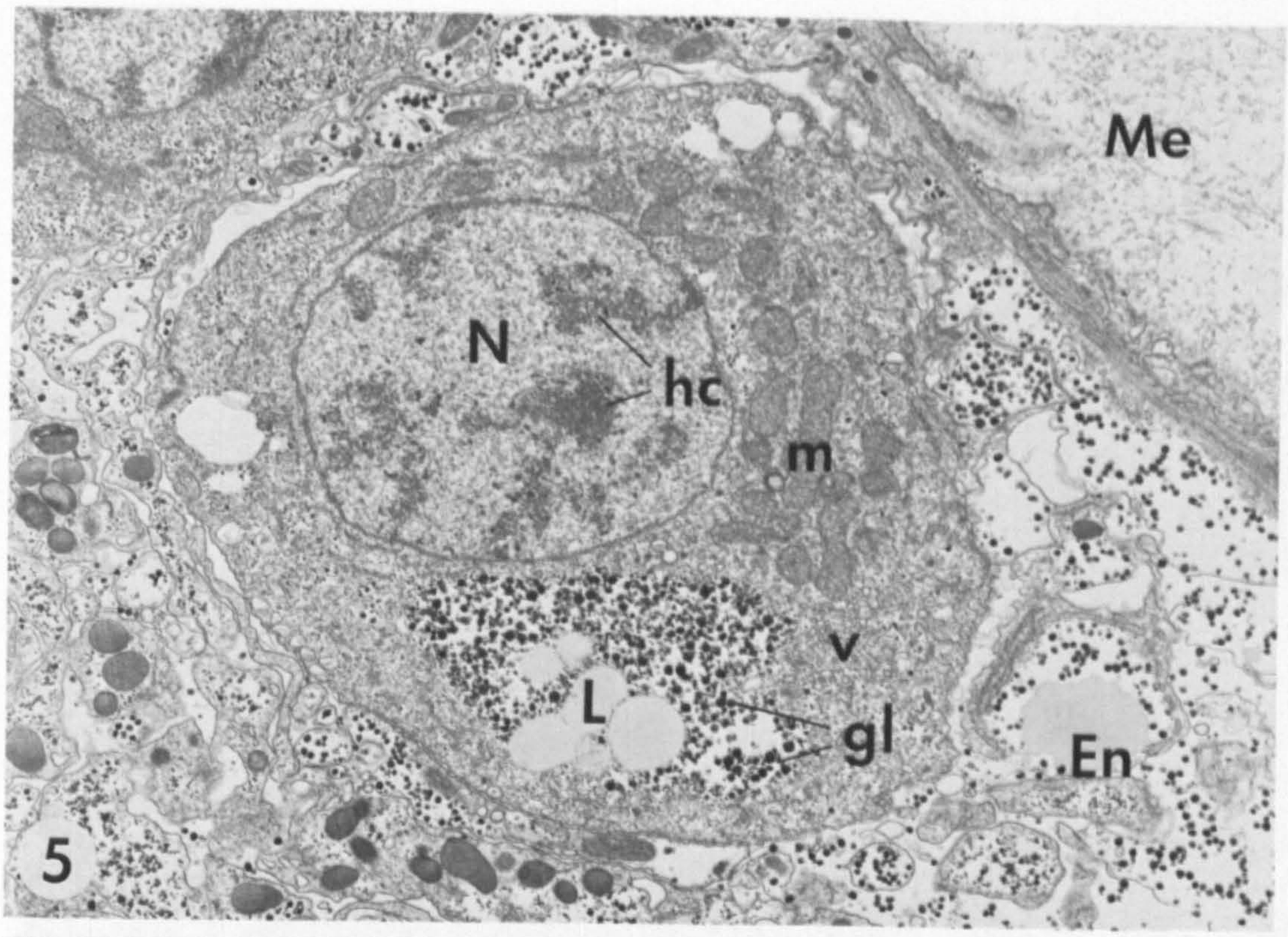
The smallest oocytes contain relatively little cytoplasm, which appears dense compared with that of the surrounding endodermal cells. Large numbers of free ribosomes are found, as well as mitochondria, Golgi complexes, glycogen, and small vesicles. As the oocytes enlarge, the amount of cytoplasm and the numbers of organelles greatly increase, but the cytoplasm retains its generally unspecialized appearance usually until the onset of vitellogenesis.

The mitochondria are usually small and rounded in section, with a dense matrix traversed by numerous cristae and may contain intramitochondrial dense granules. In the smallest oocytes many mitochondria show a characteristic latticelike arrangement of cristae, as shown in a previous paper (Larkman, '81). In oocytes over 15 μm in diameter the mitochondria are generally slightly smaller than in the smallest oocytes, averaging about 0.3 × 0.5 μm in section, and the cristae are more randomly arranged. As the oocytes grow, the number of mitochondria increases dramatically, and they often become arranged in seams or bands through the cytoplasm rather than randomly scattered (Fig. 10).

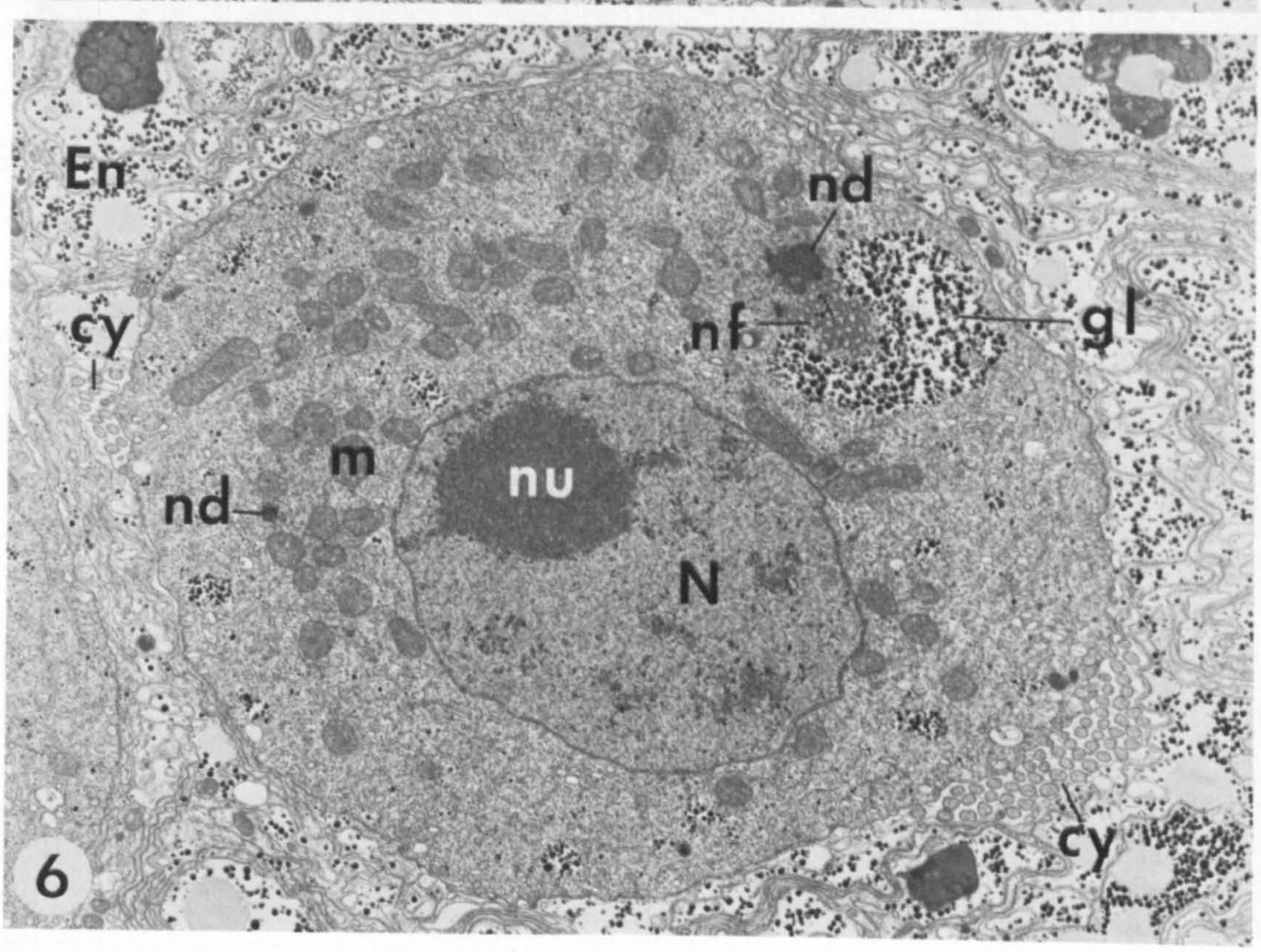
Most oocytes are seen to contain one or more Golgi complexes. These consist of parallel stacks of flattened cisternae, usually

Fig. 4. An endodermal oocyte some 13.5 μm in length, flanked by two larger mesogleal oocytes. The oocyte on the left is much larger, and is covered by a flattened amoebocyte. Note the large quantities of glycogen in the endodermal cell bases. ×11,000.


Me
Me
d
Oc
Ac
gl
m
gl
Oc
N
hc
cy
gl
mv
mp
nf
4



5



6

associated with numbers of small rounded vesicles some 40 nm in diameter. The cisternae and vesicles contain similar material of moderate electron density. The complexes are often associated with single cisternae of endoplasmic reticulum (Fig. 11). The size and number of the Golgi complexes do not markedly increase as the oocyte grows until it begins vitellogenesis.

Most oocytes accumulate considerable quantities of glycogen as they grow within the endoderm. Even the smallest recognizable oocytes contain glycogen, but it is usually present as numerous small deposits, with some glycogen particles scattered generally through the cytoplasm (Fig. 3). As the oocyte enlarges, the glycogen becomes concentrated into a smaller number of larger deposits (Figs. 4–6). These deposits may be 10 μm across in a 35-μm oocyte. The larger deposits contain proportionately more glycogen in the α-configuration than do smaller deposits. Occasionally, glycogen in large deposits appears well dispersed, and such deposits may contain empty areas and membranous vacuoles.

Lipid droplets are commonly found in small oocytes and are very often associated with accumulations of glycogen (Fig. 12). Two types of lipid droplet can be distinguished. The more common type is 0.5–1.5 μm in diameter, is homogenous, and is of low but perceptible electron density. The second type is of similar size but appears completely empty, presumably as a result of extraction of the contents during processing. While lipid droplets may be found in oocytes of all sizes, large endodermal oocytes generally appear to contain relatively less stored lipid than smaller ones.

The smallest recognizable oocytes contain little or no endoplasmic reticulum. As the oocytes enlarge, the cytoplasm comes to contain many short lengths of randomly arranged endoplasmic reticulum, some of which are studded with ribosomes. Some larger oocytes develop areas of regularly arranged endoplasmic reticulum while within the endoderm, while others may reach a considerable size (perhaps 40 μm) and may begin to accumulate yolk bodies in the apparent absence of any organized endoplasmic reticulum. By contrast, large vitellogenic oocytes in the mesoglea usually contain extensive areas of highly ordered endoplasmic reticulum.

Flagellar basal-body-rootlet complexes are often found within oocytes in the endoderm. These complexes consist of two centrioles arranged at right angles to each other connected to a striated rootlet (Fig. 13), and are similar to those found in a variety of coelenterate flagellated epithelial cells (Chapman, '74). Only one complex was ever seen in a single oocyte section. In very small oocytes (up to 15 μm) the complex may give rise to a flagellum (Figs. 1, 3; Larkman, '81), but this has not been found in larger oocytes. Structures which might represent degenerate flagella are occasionally seen. Complexes were found less frequently in larger oocytes, but this might simply reflect the lower probability of encountering a single complex in sections through the larger oocyte volume.

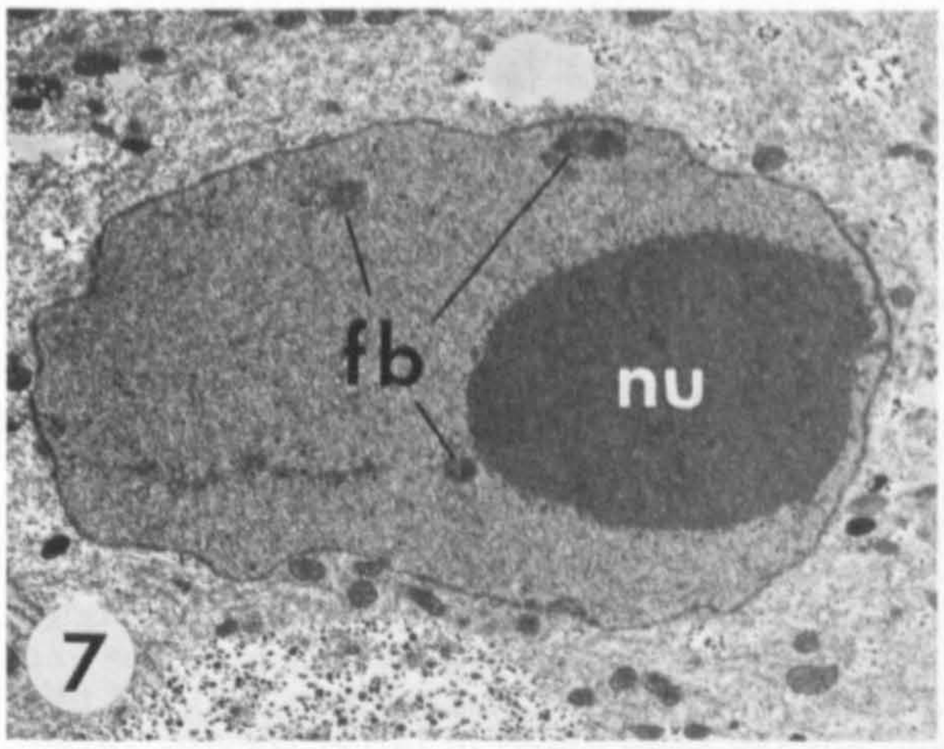
In localized regions of the oocyte, the cytoplasm may contain large numbers of membrane-bound vesicles (Figs. 14, 15). These range in size from 100 to 400 nm averaging about 200 nm in diameter. They are generally larger, less dense, and less regular than the Golgi-associated vesicles mentioned above. Many of the vesicles appear empty, while others are wholly or partly filled with material of moderate electron density. Yet others contain a smaller membrane-bound body which itself may contain granules of dense material, possibly glycogen (Fig. 13). Vesicles are often found near the oocyte surface (Fig. 14) and become especially numerous once the oocyte has begun vitellogenesis.

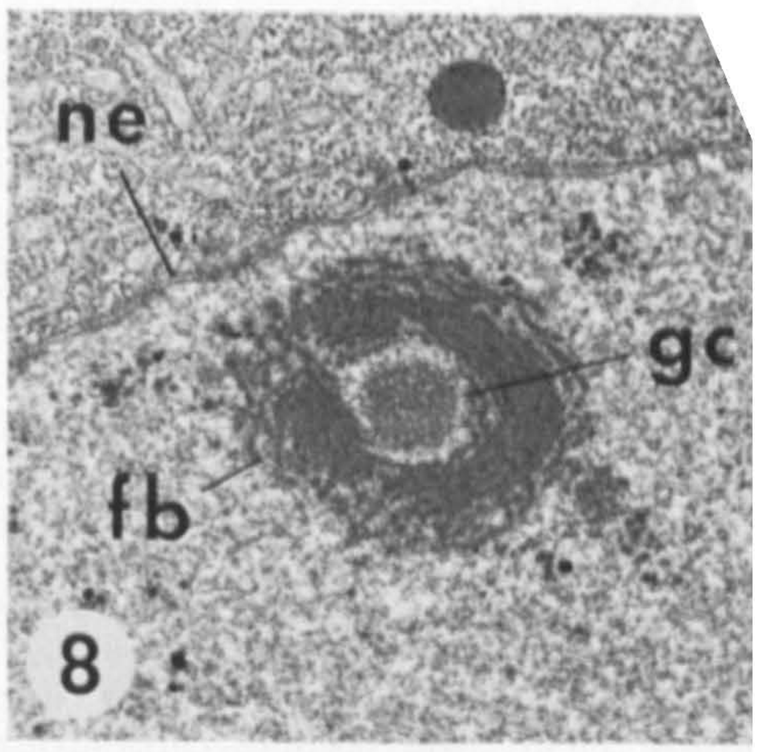
Multivesicular bodies are occasionally found in endodermal oocytes (Fig. 4). These are spherical, membrane-bound sacs, usually about 1 μm in diameter, containing numerous small vesicles, each 40–50 nm in diameter, and the occasional larger vesicle. They may occur anywhere in the cytoplasm of oocytes of any size, but are never found in large numbers.

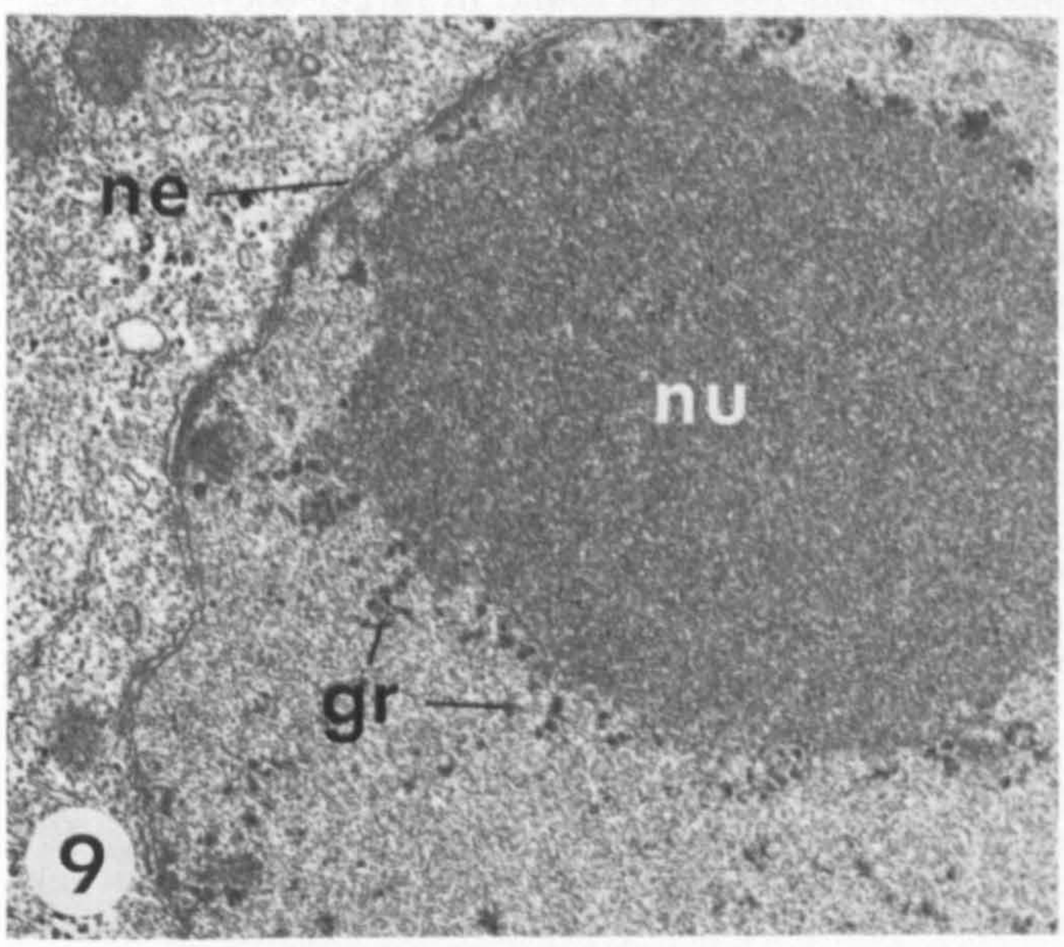
A number of apparently associated structures are found in the cytoplasm of *Actinia fragacea* oocytes which have not been observed, as yet, in any nongerminal cell types. Small oocytes (up to 12 μm) contain distinctive dense bodies which, in favourable section, resemble groups of conjoined cylinders of electron-dense material (Fig. 3). Each cylinder is about 200 nm in diameter and may

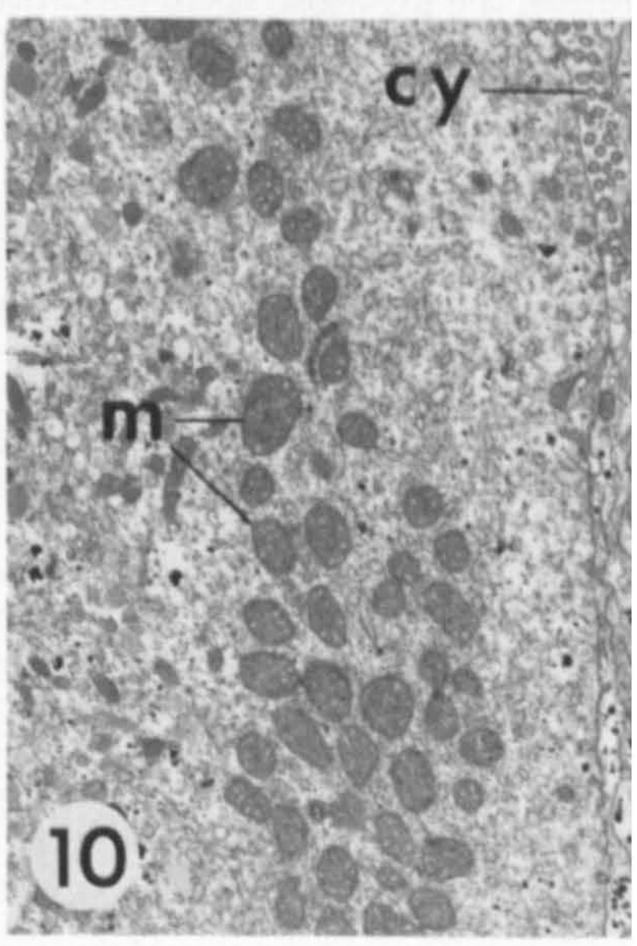
Fig. 5. A 9-μm-diameter endodermal oocyte, containing a large glycogen deposit with associated lipid droplets. ×10,500.

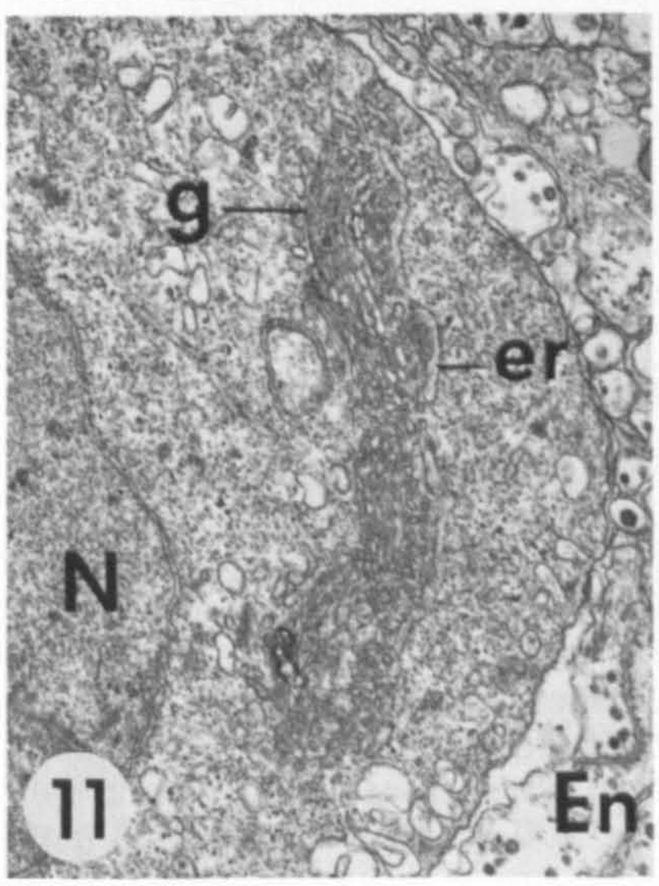
Fig. 6. A 13-μm-diameter endodermal oocyte. The nucleus is of greater density, and the surface of the cell bears groups of cytospines. ×8,500.

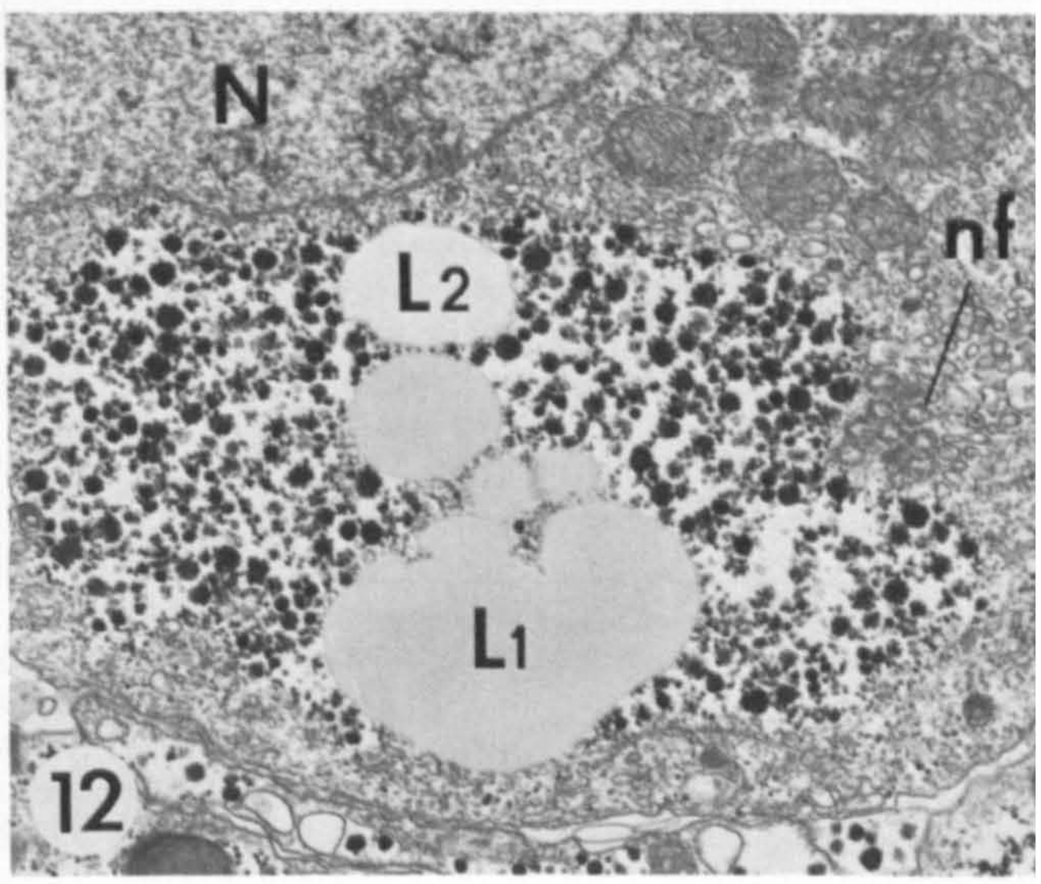

fb
nu
7


ne
gc
fb
8


ne
nu
gr
9


cy
m
10


g
er
N
11
En


N
L2
nf
L1
12

reach 0.5 μm in length. Each dense cylinder surrounds a smaller, less dense cylinder, about 100 nm in diameter, which appears to consist of a ring of longitudinal parallel fibres. These structures usually occur as clusters of three to six conjoined dense cylinders, each with its fibrillar core. Often more than one such cluster is found in a single section through an oocyte, and the clusters may be widely separated within the cell.

In larger oocytes, these clusters tend to be more extensive but less regular (Fig. 6). In particular, the fibrillar cores may be numerous and much longer, reaching 5 μm in length in some cases (Fig. 16). Many of these cores are not surrounded by dense outer cylinders, and the dense material may also be present as small fragments and larger irregular masses (Figs. 16, 17).

In larger oocytes, the clusters of cylinders are usually associated with other structures not found elsewhere in the oocyte. They are often associated with areas of dense cytoplasm containing a finely granular material (Figs. 16, 17). These dense areas are usually roughly circular in section, and may be 1–2 μm in diameter. Clusters of cylinders are also found associated with groups of membrane-lined tubules (Figs. 17, 19). These tubules average some 80 nm in diameter and are usually closely packed and roughly aligned rather than randomly arranged. Many of the tubules are curved such that circular profiles are often seen in section alongside longitudinal profiles. Additionally, groups of membrane-bound vesicles, each of which bears an external bristle coat, are sometimes found in the vicinity of the clusters of cylinders (Fig. 19). Very commonly the fibrillar cores of the cylinders extend around, and sometimes pass through, large deposits of glycogen (Figs. 4, 6, 16, 18, 19). This association was found so frequently that it is not thought to be merely fortuitous.

The cylinder clusters have also been observed in early germ cells in the male, but in this case they are not associated with any other structures. Since they have not been found in any nongerminal cell types within the anemone, these structures may represent some form of nuage material (see Discussion). It is hoped that their appearance and the changes which they undergo during gametogenesis will be described in more detail in a future publication.

Fig. 7. The nucleus of a large (>20 μm) endodermal oocyte. The nucleus contains little heterochromatin, but a large nucleolus and nuclear fibrillar bodies are now found. ×5,500.

Fig. 8. A nuclear fibrillar body with a core of finely granular material. ×21,500.

Fig. 9. The nucleolus of a large endodermal oocyte, surrounded by small nuclear granules. ×21,500.

Fig. 10. A band of mitochondria lying toward the periphery of a large endodermal oocyte. ×8,000.

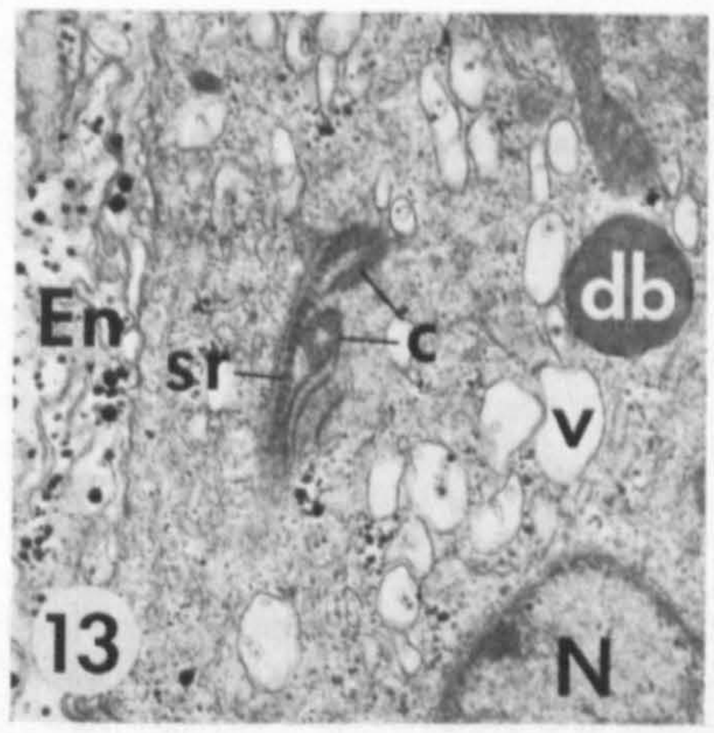
Fig. 11. A typical endodermal oocyte Golgi apparatus with associated cisternae of rough endoplasmic reticulum. ×16,500.

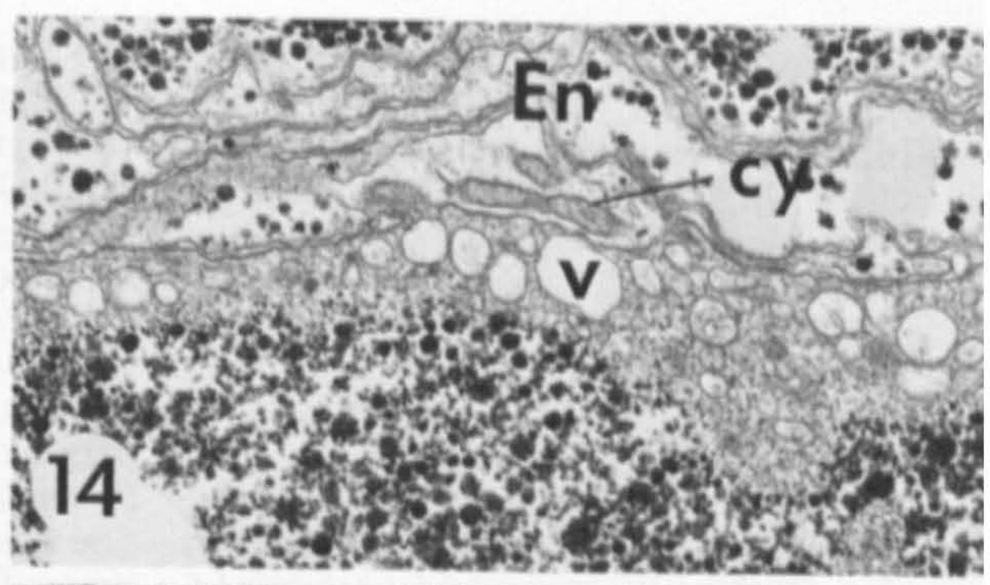
Fig. 12. A large glycogen deposit associated with lipid droplets of two types and fibrillar nuage material. ×17,500.

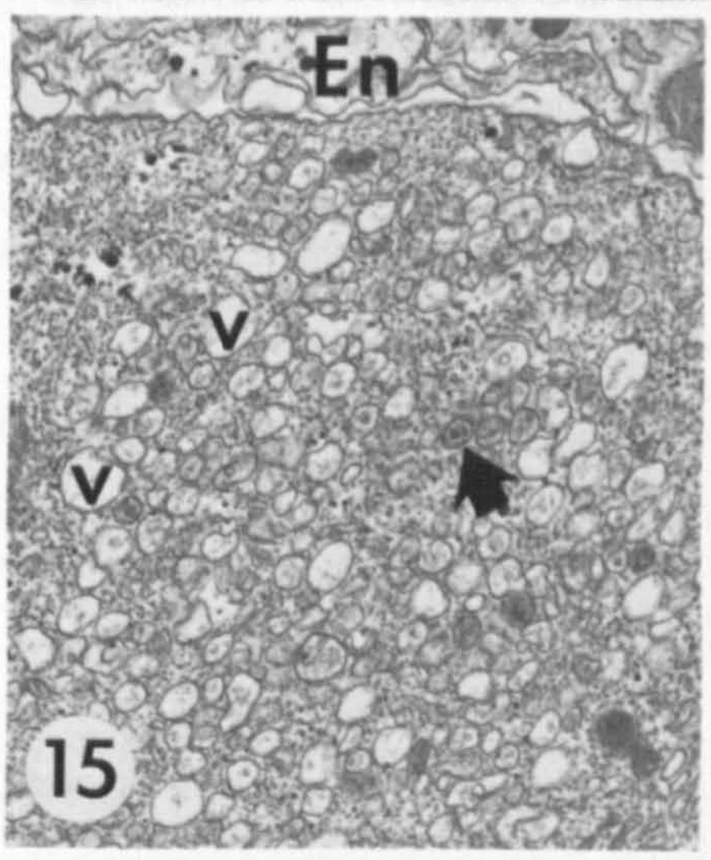
Oocyte surface changes

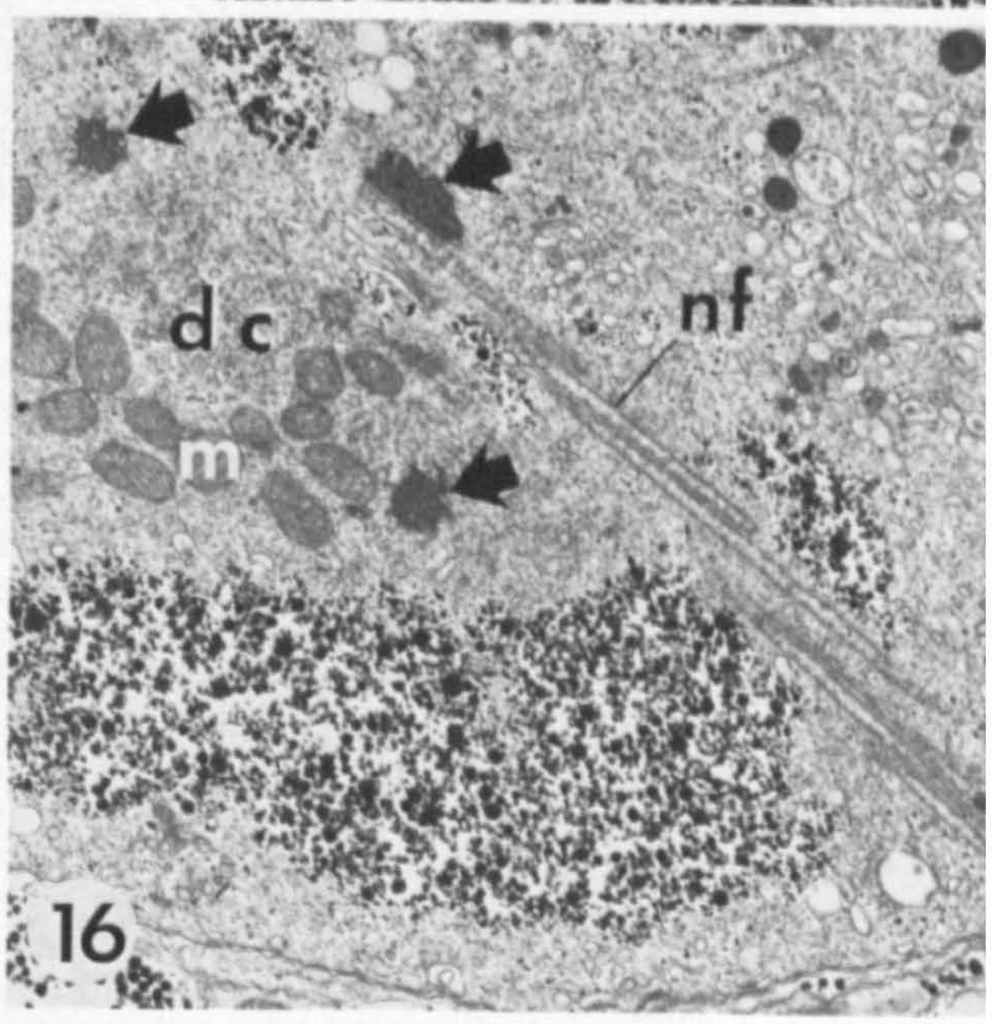
While within the endoderm the oocyte is surrounded by the basal processes of the endodermal epithelial cells. These processes vary in size and are often irregular in shape. Over most of its surface, the oocyte is separated from these processes by an irregular intercellular gap varying between 50 and 150 nm in width. Small, apparently empty, membrane-bound vesicles, 100–200 nm in diameter, which are common at this level in the endoderm, may be found in this gap. In localized areas the endodermal cell membrane closely follows the surface of the oocyte, with a narrow and rather constant intermembrane gap of about 30 nm. In these regions, desmosomelike intercellular junctions are occasionally found between the oocyte and the endoderm cell membranes (Fig. 3). Apart from these junctions, the endoderm immediately around the oocyte shows no specializations or differences from the remainder of the endoderm. At no time while the oocyte is within the endoderm is an extracellular layer visible around the oocyte plasma membrane.

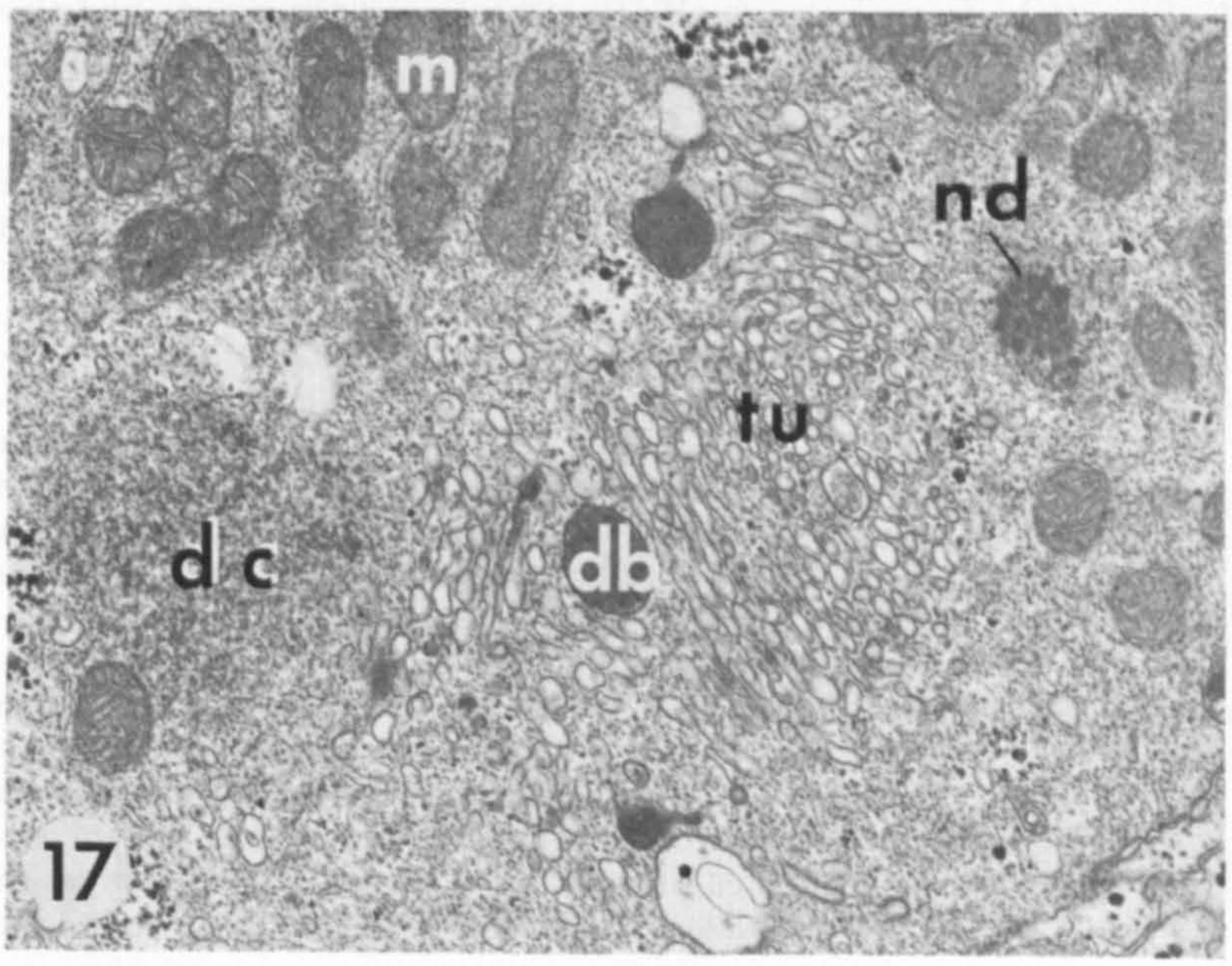
Once the oocytes reach about 12 μm in diameter, cytospines start to appear on the oocyte surface (Fig. 6). Cytospines resemble large microvilli and in small oocytes they are about 150 nm in diameter and contain a dense fibrillar core. The outer surface of the cytospine is covered with a fine glycocalyx-like material (Fig. 20). The cytospines usually lie folded flat against the surface of the oocyte. Initially the cytospines may form a single fairly evenly spaced layer over regions of the oocyte surface, but as the oocyte enlarges, the groups of cytospines become more localized and discrete, and may be two to four

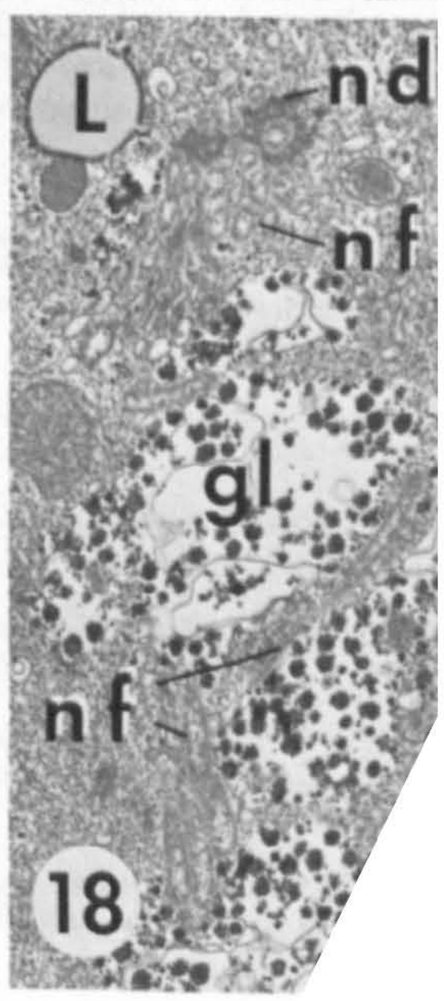

En
sr
c
db
v
13
N


En
cy
v
14


En
v
v
15


d c
nf
m
16


m
nd
tu
d c
db
17


L
nd
nf
gl
nf
18

cytospines deep (Fig. 6). Among some groups of early cytospines small bulges of the oocyte membrane are found containing material which closely resembles the contents of the cytospines (Fig. 20). These bulges may represent points of insertion of cytospines into the oocyte membrane, or might be a stage in the formation of new cytospines.

Membrane-bound vesicles, as described above, are often found near the surface of the oocyte (Fig. 14), especially near groups of cytospines. Depressions of the oocyte membrane which might be indicative of endocytosis are sometimes seen, but it is not certain that these vesicles are of endocytotic origin.

Entry into the mesoglea

After a period of growth among the basal processes of the endodermal epithelial cells, the oocytes enter the mesoglea of the gonad, where the major part of oocyte growth and vitellogenesis takes place. There does not appear to be a specific size or stage of development at which oocytes enter the mesoglea. Some oocytes migrate into the mesoglea at a diameter of about 12 μm, while others remain in the endoderm until they reach 40 μm and occasionally more. Since the development of oocytes even within a single gonad may be far from synchronous, some small oocytes may enter areas of mesoglea already densely populated by much larger oocytes.

The mesoglea of the gonad in *A. fragacea* is similar in appearance to that of the rest of the mesentery, although the collagen fibrils are generally less numerous and less regularly arranged. The mesoglea is usually flanked on both sides by muscle processes from the endodermal epithelial cells, although their degree of development varies. Often they form a thin continuous layer and are linked to each other by desmosomelike junctions (Figs. 1, 4). These junctions resemble the septate desmosomes described for sea anemone endoderm by Green and Bergquist ('82), although the septae are not obvious in the conventionally stained material used in the present study. A thin basal lamina always separates the endodermal processes from the mesoglea.

Most oocytes appear to make their way into the mesoglea by a process resembling amoeboid movement. However, the possibility that the endodermal cell bases actively contribute to the movement of the oocytes cannot be ruled out. Prior to entry, some small oocytes already rest against the mesoglea, and others may contact it by means of slender cytoplasmic processes (Larkman, '81). For most oocytes, however, the first step of entry into the mesoglea appears to be to open a gap in the endodermal musculature. A short process is sent out from the surface of the oocyte which penetrates the musculature and makes contact with the mesoglea. This oocyte process does not appear to be specialised in any way. The process then pushes into the mesoglea, but in doing so it does not puncture the basal lamina, which thus coats its tip (Fig. 21). The oocyte now flows through the gap in the musculature and progressively passes into the mesoglea, carrying the basal lamina forward as it does so. As the portion of the oocyte within the mesoglea enlarges, the oocyte remains constricted at the point where it passes through the muscle layer, giving the oocyte a characteristic "hourglass" or "dumbbell" shape which can easily be recognised in light microscope sections. With small oocytes, the nucleus is usually positioned near the leading edge of the cell, and so enters the mesoglea relatively early (Figs. 22, 24). With larger oocytes (above 15 μm in diameter), the nucleus usually occupies a more central position (Figs. 23, 25).

When small (less than 15 μm) oocytes enter the mesoglea, their leading edge tends to be smooth and rounded (Fig. 22). With larger oocytes, the initial oocyte process may project some distance into the mesoglea before it begins to fill out (Fig. 23). When the process does fill out, its leading edge often bears pro-

Fig. 13. Part of the cytoplasm of an endodermal oocyte containing a basal-body-rootlet complex and numerous vesicles, some of which enclose central cores containing dense material. ×15,500.

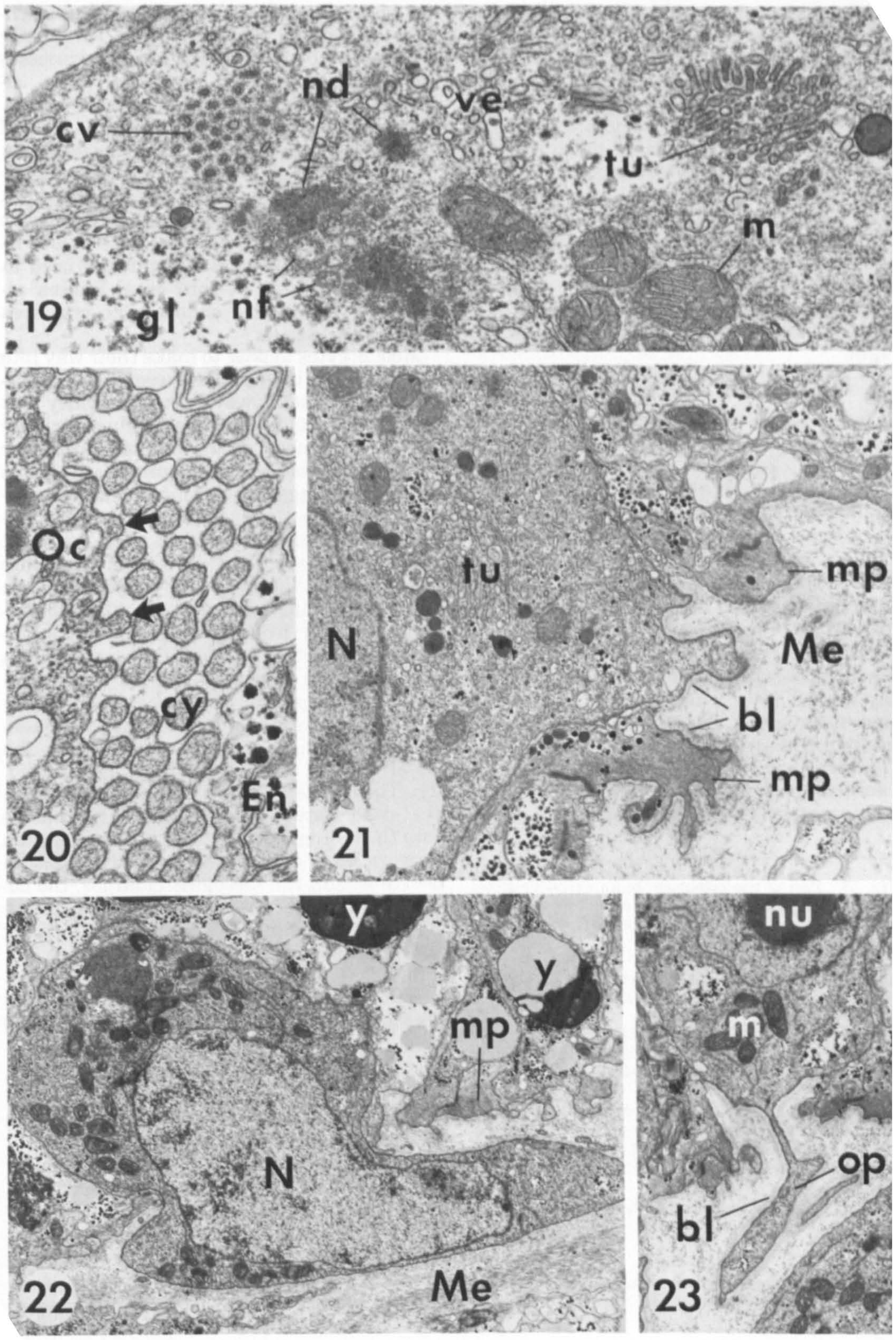
Fig. 14. Superficial region of oocyte, showing a row of vesicles just below the surface. ×18,000.

Fig. 15. A region of an oocyte containing many small vesicles. Some vesicles appear empty, while others (arrowed) contain dense material. ×18,500.

Fig. 16. Part of an endodermal oocyte showing three areas of dense nuage material (arrowed) and several long cores of fibrillar nuage. An area of dense cytoplasm is associated with several mitochondria. ×12,000.

Fig. 17. A region of ooplasm containing dense nuage, membranous tubules and an area of dense cytoplasm. ×18,500.

Fig. 18. A glycogen deposit closely associated with dense and fibrillar nuage material. ×11,500.


nd
ve
cv
tu
m
19
gl
nf
Oc
cy
En
20
tu
mp
N
Me
bl
mp
21
y
y
mp
N
Me
22
nu
m
bl
op
23

jections which may facilitate further penetration of the mesoglea (Fig. 24). Indeed, the larger the oocyte, the more irregular in outline does the portion within the mesoglea become (Fig. 26). Not only may its leading edge be indented, but its lateral surfaces may be deeply invaginated such that regions of the oocyte appear discontinuous in section. Many of these invaginations may contain cytospines, and the constricted "neck" region of the oocyte may appear vacuolated. The basal lamina covers the entire surface of the oocyte within the mesoglea, closely following all its irregularities, and can be seen to be continuous with the basal lamina of the neighbouring epithelial cells (Figs. 21–27).

When most of the oocyte has entered the mesoglea, it may become even more severely constricted at the muscle layer such that the portion remaining in the endoderm may be connected to the rest of the oocyte by only a slender neck (Fig. 25). The constriction may be less severe than suggested by this micrograph since this section may not pass through the widest part of the neck. Oocytes can apparently remain with a small portion left in the endoderm for a considerable time. The possible significance of this continued contact with the endoderm will be discussed below.

While it appears that most oocytes actively force their way into the mesoglea as described above, it is possible that in some instances the mesoglea may grow out around the oocyte, at least to some extent. Sometimes indications of mesogleal outgrowth are seen around the oocyte process as it passes through the gap in the muscle layer (Figs. 27, 29). Whether this is a result of new mesogleal material being formed around the oocyte or whether existing material is pulled out of position as the oocyte enters is unclear. Some such mechanism may be important when large oocytes enter relatively narrow layers of mesoglea.

Occasionally "spurs" or side branches extend out from the main layer of gonad mesoglea, and may make contact with oocytes in the endoderm. Whether such contacts are the result of directed mesogleal outgrowth, directed oocyte migration, or are merely fortuitous is uncertain.

Maintenance of oocyte/endoderm contact

Stages similar to that shown in Figure 25, in which most of the oocyte is within the mesoglea but a small portion remains in the endoderm, were encountered very frequently, suggesting that this situation might be relatively stable and long-lasting. A more detailed examination of oocytes at this stage revealed a number of interesting features.

The small portion of oocyte remaining in the endoderm appears to enter into a close relationship with the bases of the endodermal cells surrounding it. Extensive intercellular junctions often form between the oocyte and the endodermal cell membranes (Fig. 28). The neck region of the oocyte becomes indented, and endodermal cell bases and muscle processes are closely applied to its surface and follow its contours. The muscle processes often project out from the general line of the endoderm/mesoglea boundary as though they have grown or been dragged into the mesoglea by the entering oocyte. Again, desmosome-type junctions, similar to those found between adjacent muscle processes, link the muscle processes to the neck region of the oocyte.

Later in the formation of the oocyte/endoderm relationship, the surface of the endodermal portion of the oocyte may become densely covered with large cytospines (Fig. 30), which project into and between the endodermal cell bases and form a region of intimate contact between oocyte and endoderm. The localization of the cytospines may be quite precise. In Figure 30, cytospines are virtually absent from the neck region of the oocyte, and from the region of contact with the muscle processes, where intercellular junctions form. However, they are large and closely packed where the oocyte makes contact with the unspecialised endoderm bases, which contain large amounts of lipid and glycogen.

A ring-shaped depression often forms around the base of the neck region in the

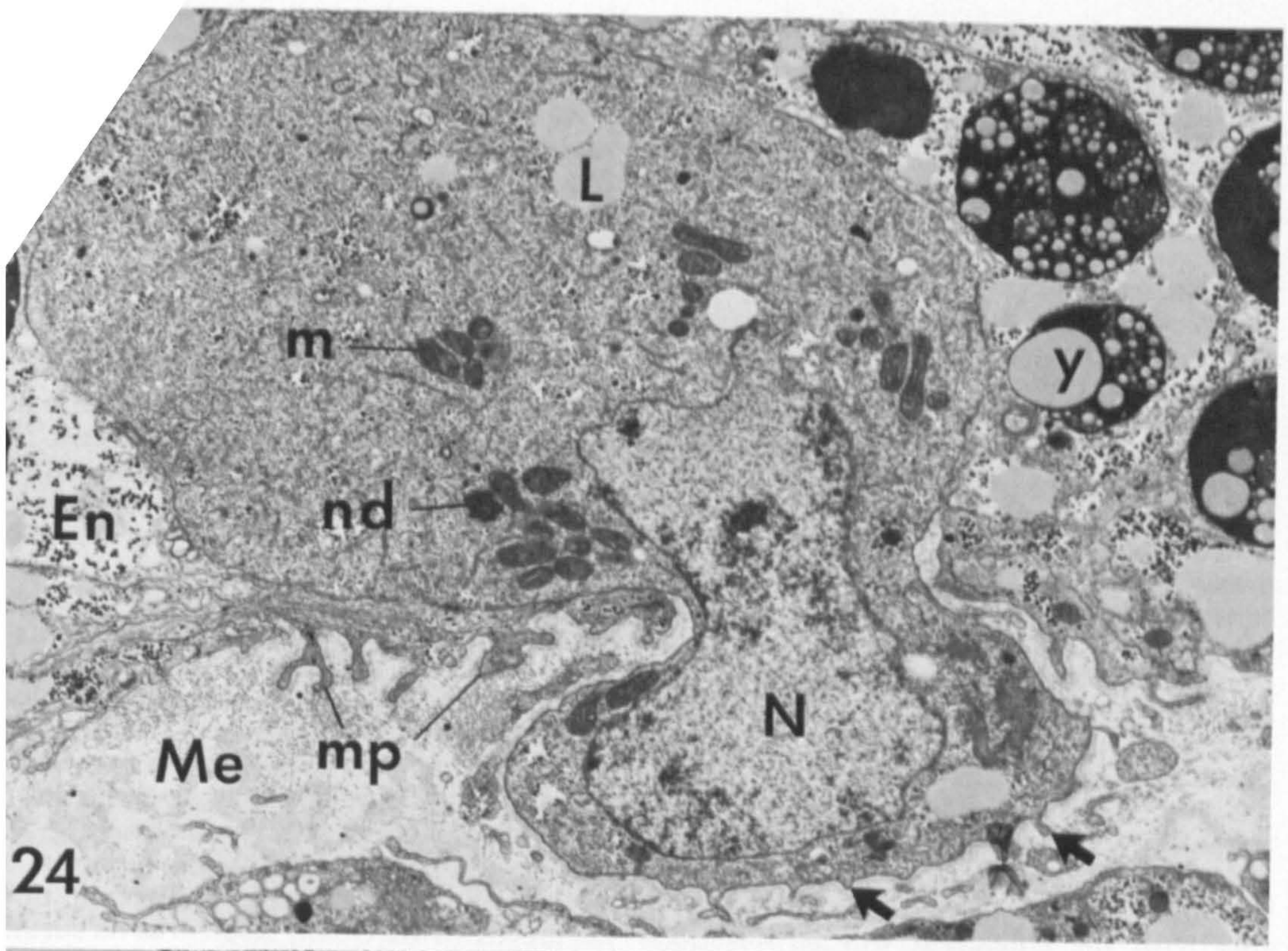
Fig. 19. An area of nuage associated with glycogen, vesicles, mitochondria, and tubules. ×31,000.

Fig. 20. Group of transversely sectioned cytospines. The oocyte surface bears budlike outgrowths (arrowed). ×35,000.

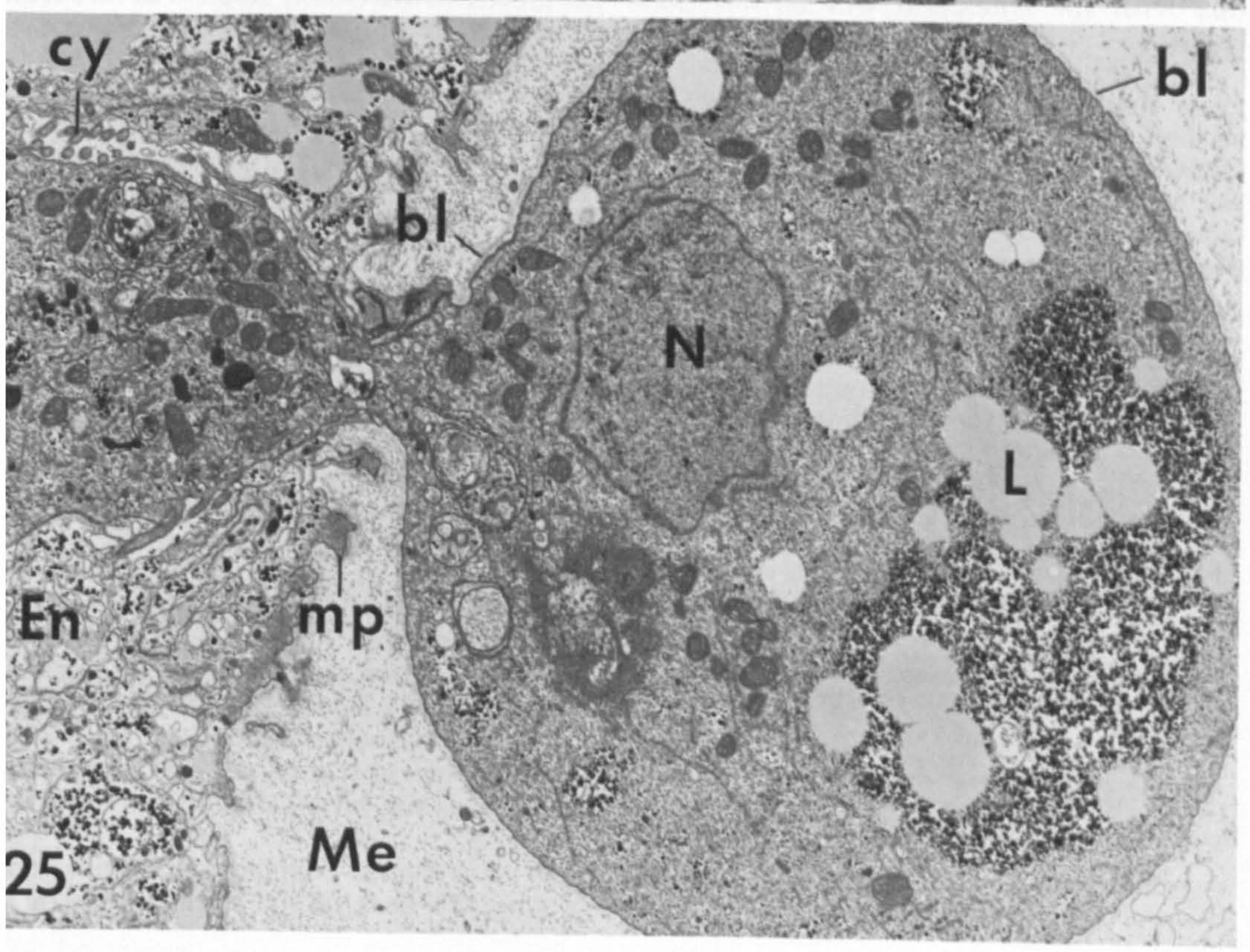
Fig. 21. Oocyte just beginning entry into the mesoglea. The tip of the oocyte is covered by the basal lamina. ×10,500.

Fig. 22. A small (11-μm) oocyte during entry into the mesoglea. The endodermal cells contain yolk granules from large oocytes undergoing resorption. ×6,000.

Fig. 23. A 15-μm oocyte which has sent a slender process into the mesoglea prior to entry. ×5,700.



24



25

surface of the mesogleal portion of the oocyte. This depression contains large numbers of cytospines and cytoplasmic processes from the endodermal cells which have presumably grown or been pulled into the mesoglea with the oocyte. These processes may contain mitochondria, lipid droplets, and often large quantities of glycogen (Fig. 31). They may extend between the cytospines and contact the oocyte surface, and again may participate in desmosome formation. Away from the base of the depression, around the neck of the oocyte, the endodermal processes contain numerous microfilaments usually aligned along the axis of the oocyte neck (Fig. 31). The oocyte cytoplasm beneath areas of close contact between the oocyte surface and the endoderm contains large numbers of small, membrane-bound vesicles (Figs. 30, 31). These vesicles are similar in appearance to those found in the cytoplasm next to the region of oocyte/endoderm contact in the trophonema of larger oocytes (Larkman and Carter, '82). The main features of this stage of oocyte entry are summarized in Figure 32.

Eventually the remaining endodermal portion of the oocyte is pulled into the mesoglea with the rest of the oocyte, and the ring-shaped depression in the surface of the oocyte becomes cup-shaped. However, contacts between the oocyte and the processes from the endodermal cells are not severed. These contacts are thought to play an important part in the formation of the trophonema (see Discussion) and hence in the nutrition of the growing oocyte.

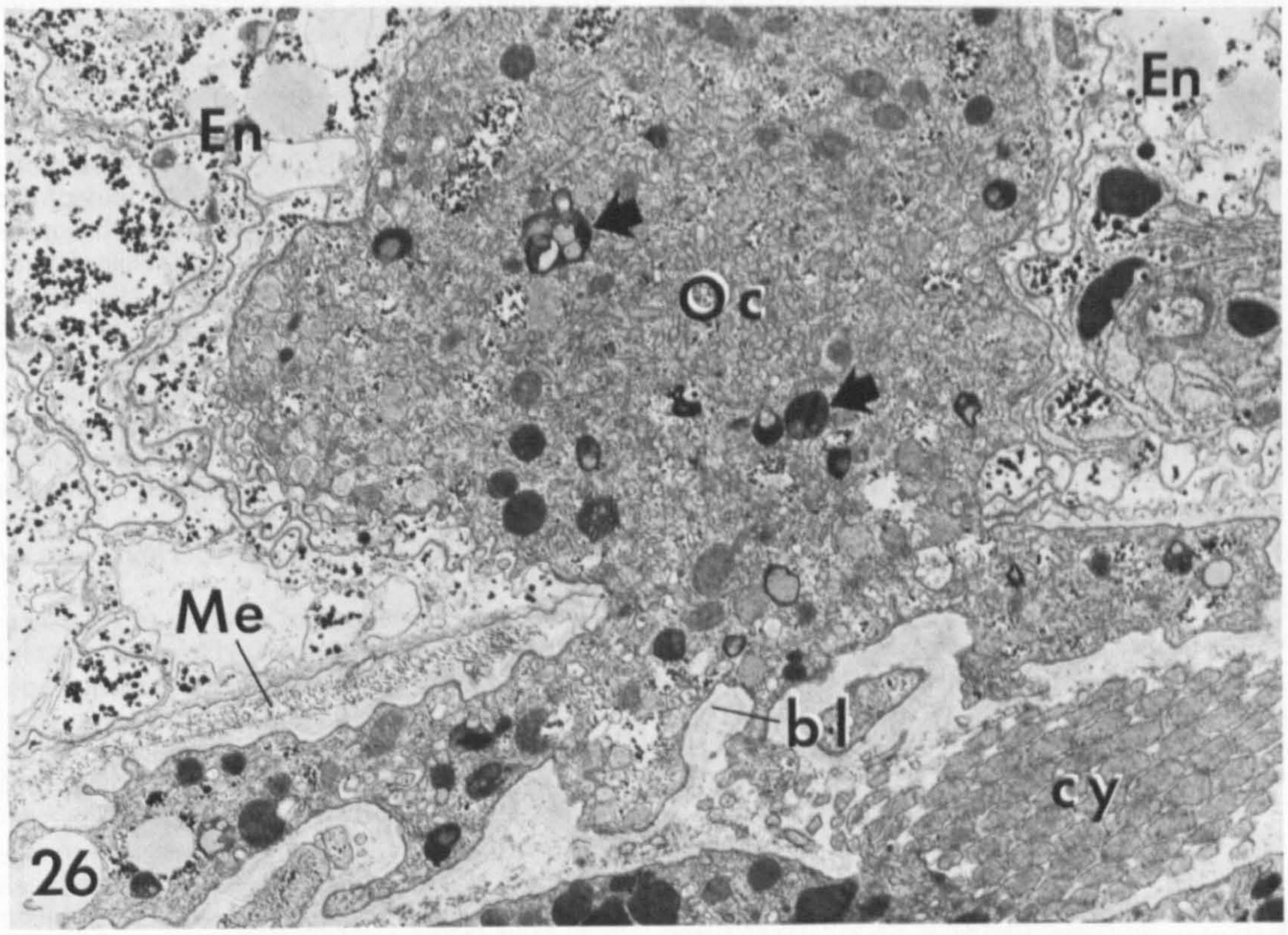
DISCUSSION

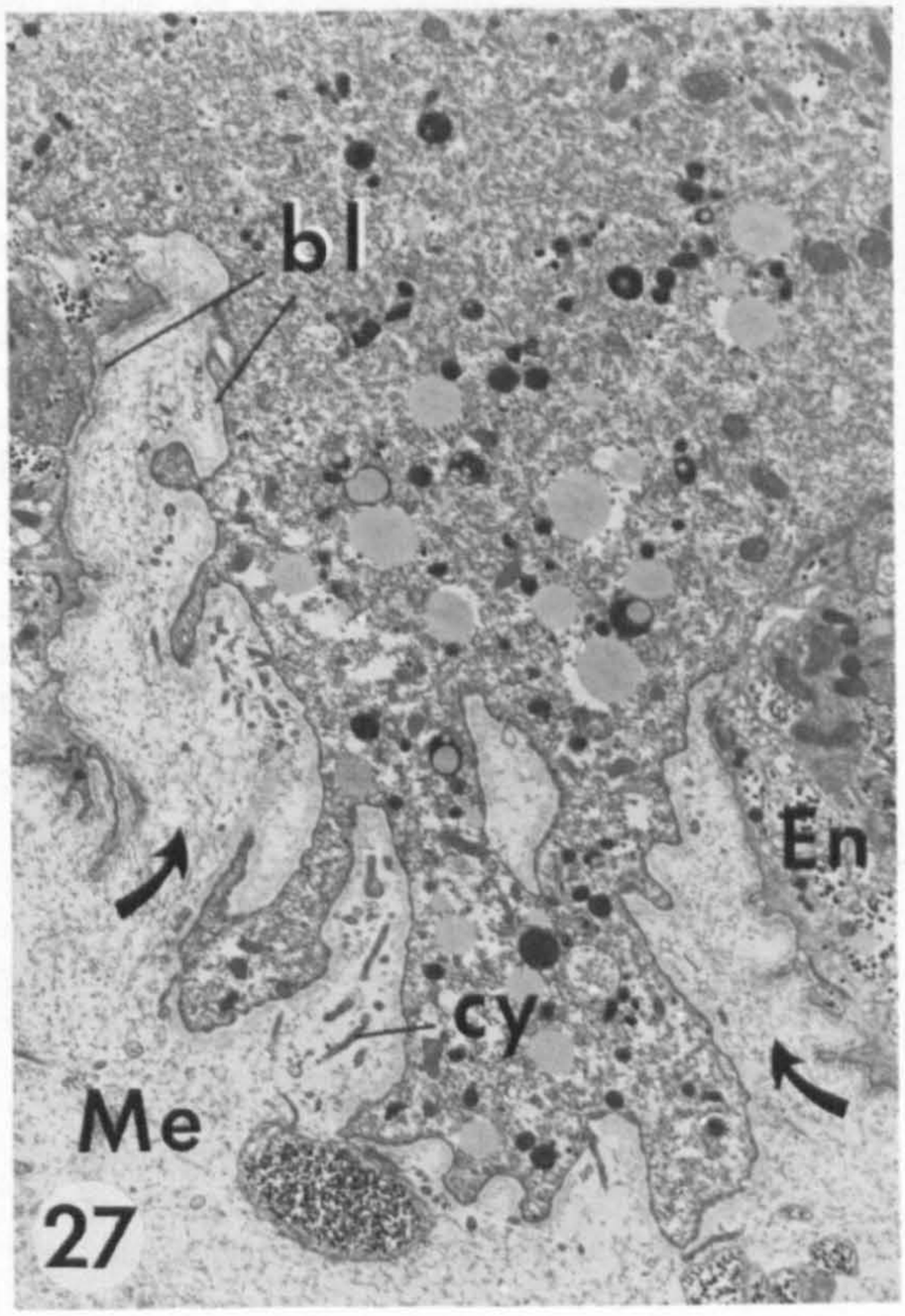
The precise cellular origin of the germ cells in *A. fragacea* is unclear (see Larkman, '81). Most recent authors agree that anthozoan germ cells are of endodermal origin (Dunn, '75; Jennison, '79, '81; Schäfer and Schmidt, '80; Szmant-Froelich et al., '80; Nieuwkoop and Satasurya, '81). This contrasts with the situation in *Hydra*, where the gametes derive from interstitial cells located in the ectoderm (Zihler, '72; Tardent, '74). The above anthozoan workers also suggest that anthozoan germ cells derive from interstitial cells. The earliest recognizable female germ cells in *A. fragacea* could be termed interstitial cells on the basis of their ultrastructural appearance, being small, rounded cells with relatively large nuclei and scant, basophilic cytoplasm containing many free ribosomes (Larkman, '81). However, it is not clear that pluripotent stem cells which are capable of differentiating into cell types other than germ cells exist in adult sea anemones. Until more is known about the developmental capabilities of these cells in sea anemones, it might be prudent to restrict the term interstitial cell to those cells which are known to be pluripotent, rather than use it for any class of cells which seem morphologically unspecialized.

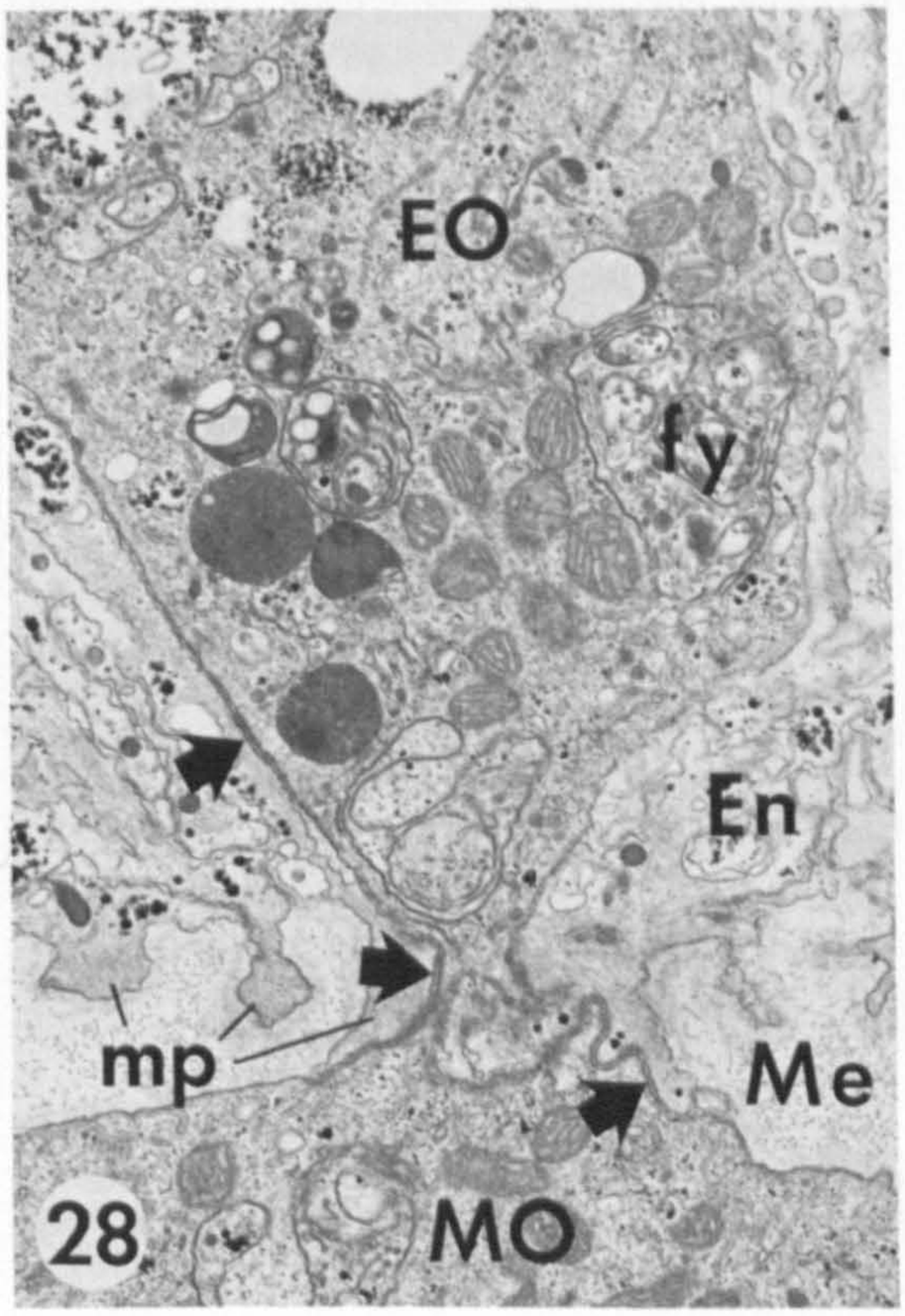
The early, endodermal oocytes of *A. fragacea* share many characteristics with the previtellogenic oocytes of many animal species (Norrevang, '68; Anderson, '74). In general appearance they resemble the early oocytes described by Schäfer and Schmidt ('80) and Schmidt and Schäfer ('80) from various anthozoan species. However, there appears to be a number of detail differences between early *A. fragacea* oocytes and the generalized descriptions given by Schmidt and Schäfer. The outline of the endodermal oocytes of *A. fragacea* is generally smooth with only small indentations, some of which may be concerned with pinocytosis. Their surface was not observed to be enlarged by manifold indentations containing glycogen-like material as well as many small vesicles, as indicated by Schmidt and Schäfer. They also suggest that small endodermal oocytes are surrounded by many free vesicles and glycogenlike granules. In *A. fragacea* the oocytes are closely enveloped by the basal processes of the endodermal epithelial cells. These processes may contain lipid droplets and glycogen, and small vesicles are often found amongst them. However, these vesicles are not more numerous in the vicinity of small oocytes than elsewhere in the endoderm. Schmidt and Schäfer also state that there is a loose vitelline membrane of fine granular material on the surface of young oocytes still in the endoderm. In *A. fragacea* there is no extracellular layer of any description visible around the oocyte while it is within the endoderm. The oocyte plasma membrane often contacts the membranes of the endodermal cell bases closely, with only

Fig. 24. A larger, 20-μm oocyte in the process of entering. The leading edge of the oocyte bears projections (arrowed) which may aid penetration of the mesoglea. ×5,500.

Fig. 25. A 20-μm-diameter oocyte which has nearly completed entry. Note the severe constriction of the oocyte and the cytospines on the endodermal portion. ×7,500.


En
En
Oc
Me
bl
cy
26


bl
En
cy
Me
27


EO
fy
En
mp
Me
28
MO

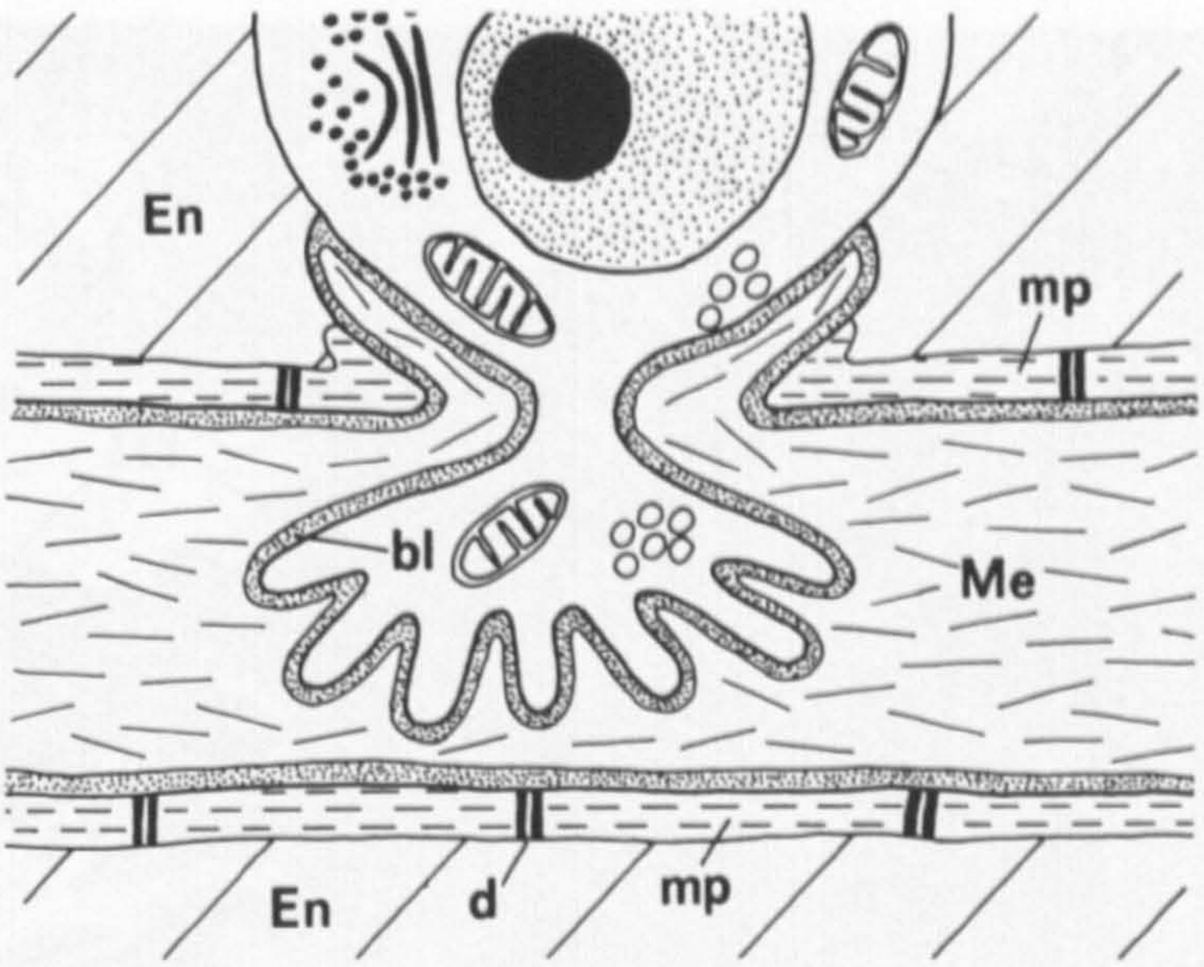


Fig. 29. Summary diagram illustrating the entry of relatively large (> 15-μm-diameter) oocytes into the mesoglea. The oocyte is constricted at the endoderm/mesoglea boundary, and its leading edge is deeply indented. The mesoglea is shown apparently growing or being pulled out around the neck of the oocyte, and the basal lamina covers the mesogleal portion of the oocyte.

a narrow intermembrane gap, and desmosomes are sometimes formed between the two. As the oocytes enter the mesoglea, they do become surrounded by a layer of extracellular material. Whether or not this layer should be regarded as a vitelline membrane will be discussed below.

Schäfer and Schmidt ('80) suggest that ribosomes appear in the ooplasm during oocyte migration into the mesoglea. In *A. fragacea*, however, the ooplasm contains numerous ribosomes from the earliest recognizable stages (Larkman, '81). Kessel ('68) found numerous free ribosomes in the early oocytes of a hydrozoan medusa, and their abundance is a characteristic feature of many unspecialized cells and primordial germ cells (Nieuwkoop and Satasurya, '81).

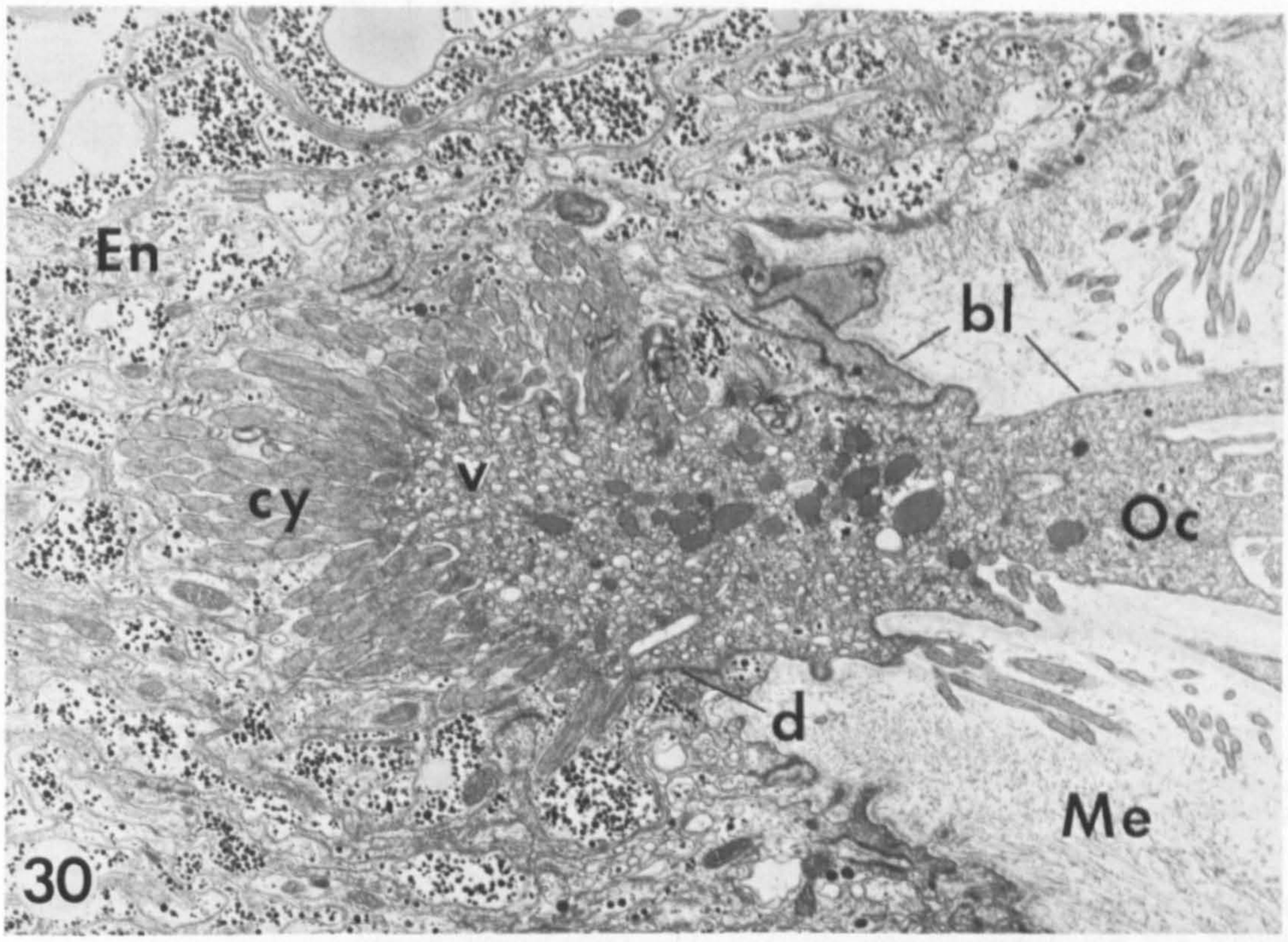
The progressive increase in nuclear density seen in *A. fragacea* oocytes as they grow within the endoderm has not been remarked by other anthozoan workers. The nucleus increases in electron density until the phase of rapid nuclear enlargement which occurs early during vitellogenesis, after the oocyte has entered the mesoglea. The density of the nucleus then falls and is generally low in all oocytes over about 50 μm in diameter (unpublished observation).

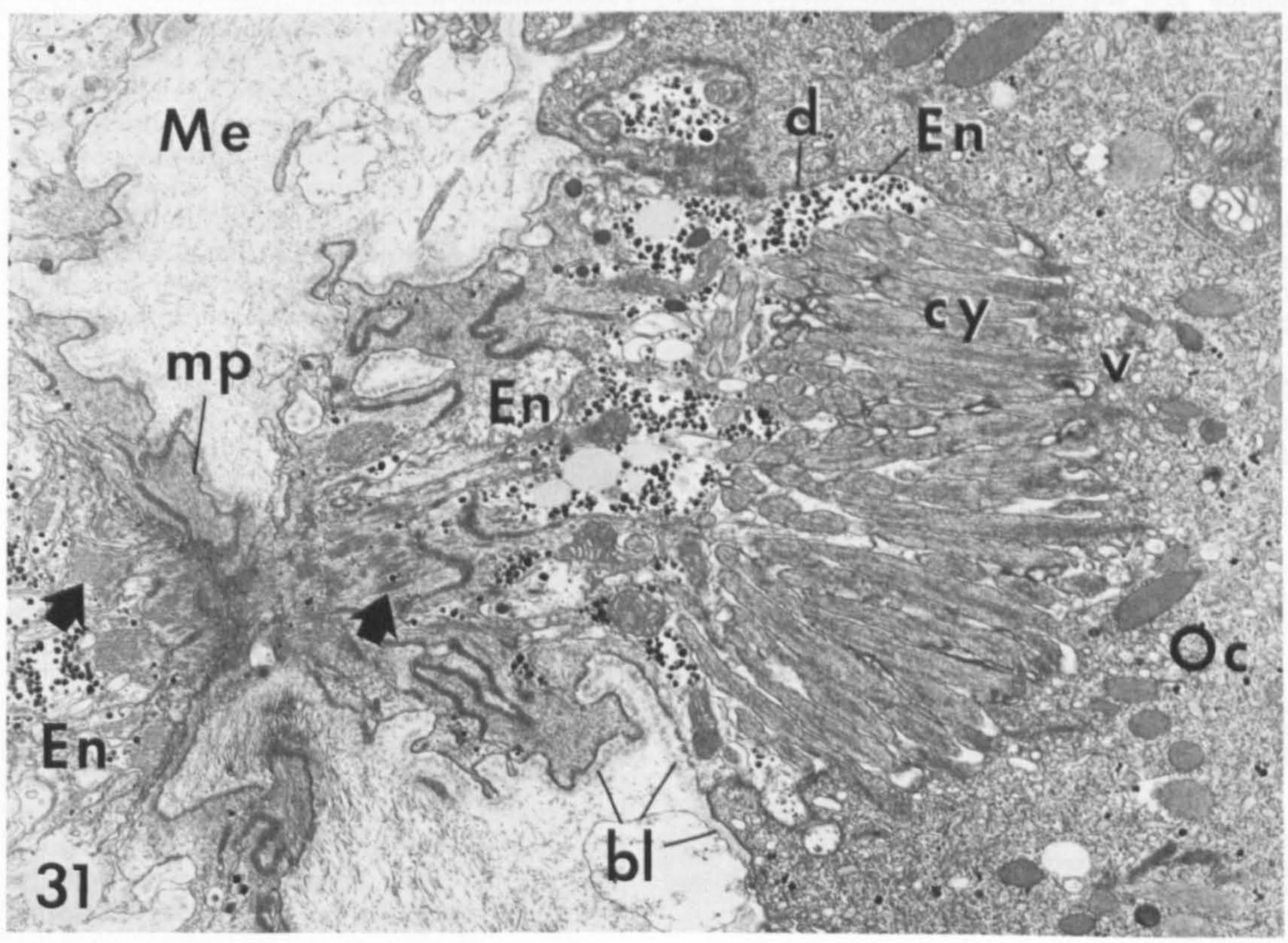
The term cytospine was introduced by Dewel and Clark ('74) to clarify a confusing situation which arose when a structure previously described and named using the light microscope was observed by electron microscopy. Several earlier light microscope investigators had observed projections from the surface of anthozoan oocytes which were generally referred to as spines (Gemmill, '20, '21; Chia and Rostron, '70; Spaulding, '72; Chia and Spaulding, '72). With the electron microscope, Dewel and Clark ('74) found that in the sea anemone *Bunodosoma cavernata* the spines were actually bundles of smaller projections, each of which resembled a large microvillus. They proposed that each of the smaller projections be termed a cytospine,

Fig. 26. A relatively large oocyte, containing several yolk granules (arrowed), spreading laterally as it enters an area of mesoglea already occupied by a much larger oocyte bearing tufts of cytospines. ×6,400.

Fig. 27. Part of a relatively large oocyte entering the mesoglea, showing a thin layer of mesoglea (curved arrows) growing or being pulled out around the oocyte as it enters. ×4,500.

Fig. 28. The neck region between endodermal and mesogleal portions of an oocyte which has nearly completed entry. Intercellular junctions (arrowed) have formed between the oocyte and the endodermal cell bases. ×10,000.


En
bl
cy
v
Oc
d
Me
30


Me
d
En
cy
mp
En
v
Oc
En
bl
31

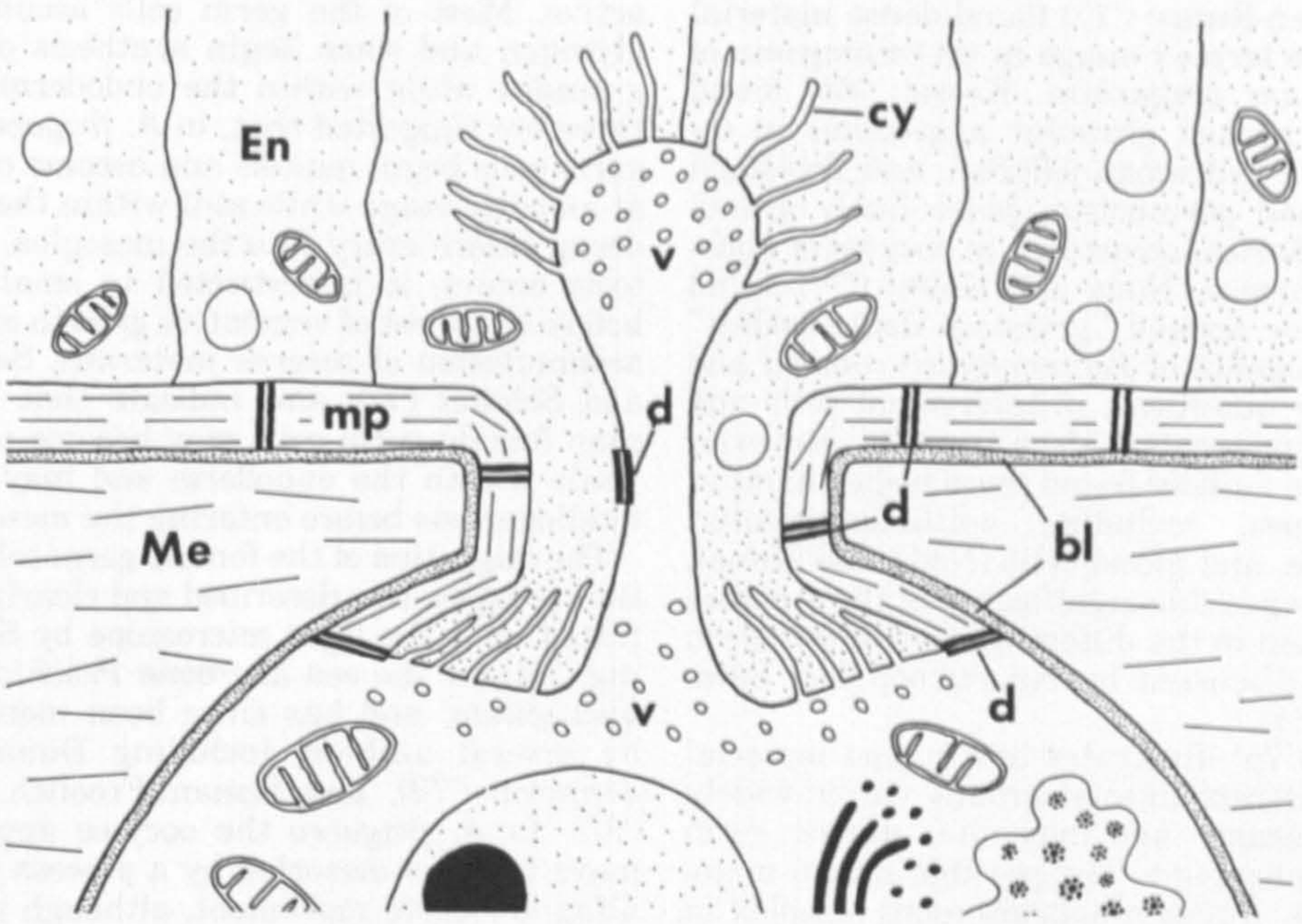


Fig. 32. Summary diagram illustrating the relationship between oocyte and endoderm just before mesogleal entry is completed, at a stage similar to that shown in Figures 30 and 31. Endodermal cell bases extend around the neck of the oocyte and terminate in a ring-shaped depression in the mesogleal portion of the oocyte.

and their suggestion has been followed in the present paper. Cytospines have since been observed in many anthozoan species (Schmidt and Schäfer, '80).

The surface of the fully grown *A. fragacea* oocyte is covered by cytospines arranged in definite tufts (Larkman, '80) and the beginnings of this organization can be seen in larger endodermal oocytes. In large mesogleal oocytes the fibrillar core of the cytospine is more obvious and projects from the base of the cytospine as a rootlet (Spaulding, '74; Larkman, '80). Such rootlets were not observed in endodermal oocytes. Schmidt and Schäfer consider the cytospines to be resorptive organelles, and the present observations on small oocytes of *A. fragacea* are consistent with this function. Cytospines clearly increase the surface area of the oocyte and may make close contact with the surrounding endodermal cells. Small vesicles, which may be pinocytotic in origin, are more numerous in oocytes which bear cytospines and tend to be more numerous in the vicinity of groups of cytospines. Microvilli, which cytospines closely resemble, are thought to be involved in nutrient uptake in many other oocytes (e.g., Anderson, '74; Schade and Shivers, '80; Eckelbarger and Grassle, '82). Cytospines may also be involved in the formation of the trophonema (see below).

The clusters of electron-dense structures found in *A. fragacea* oocytes have also been found in early male germ cells (unpublished observation) but as yet have not been seen in cells of any other type. This raises the possibility that these structures may correspond to the nuage material described from many germ cells (Eddy, '75). Roosen-Runge ('77) describes nuage as "the dense fibrous material (order of magnitude 1 μm or less) seen in germ cells of many animals," a description which could be applied to these structures. Nuage material has not previously been reported from other sea anemone germ cells, although I have observed very similar structures in *Cereus pedunculatus* oocytes (unpublished observation). Nuage has been reported in germ cells of several hydrozoans howev-

Fig. 30. The small endodermal remnant of an oocyte now largely within the mesoglea. ×8,000.

Fig. 31. A depression in the mesogleal portion of an oocyte at a similar stage to that in Figure 30. This section misses the endodermal remnant of the oocyte, but passes through the endodermal cell bases around it, which contain bundles of microfilaments (arrowed). ×10,000.

er. Roosen-Runge ('77) found dense material which he termed nuage in spermatogonia of *Phialidium gregarium*. Kessel ('68) found densely packed granular aggregates in oocytes of a hydrozoan jellyfish, and Boelsterli ('77) found perinuclear dense finely granulated spherical structures in oocytes of *Podocoryne carnea*. Noda and Kanai ('77) found what they termed "germinal dense bodies" in the oogonia of *Pelmatohydra robusta* and in lesser quantities in interstitial cells and early nematocytes. More recently, however, the same authors found these bodies in other cell types, including epitheliomuscular, digestive, and gland cells (Noda and Kanai, '80). The possible significance of these bodies in relation to the differentiation of the germ cells is discussed by Nieuwkoop and Satasurya ('81).

Eddy ('75) illustrates how nuage material from different animal groups varies widely in appearance and may alter during germ cell development. The possible nuage material in *A. fragacea* appears more complex in structure than that described for other coelenterates and most other groups. In very small oocytes (less than 12 μm diameter) the nuage is rather regular and consistent in form, but in larger oocytes it tends to be more extensive, more variable in appearance, and often associated with other organelles. However, whether this material plays any part in the determination of the germ cells in *A. fragacea* remains to be demonstrated.

In *Epiactis prolifera*, Dunn ('75) suggested that primary oogonia range in size from 6.0 to 8.5 μm in diameter while germ cells in the endoderm up to 30–35 μm are secondary oogonia. The germ cells cease dividing and become oocytes once they are within the mesoglea. Jennison ('79) describes a similar situation in *Anthopleura elegantissima*. In the present study, however, no evidence of mitotic activity was seen in any of the larger germ cells. Endodermal germ cells as small as 10-μm diameter contained synaptinemal complexes, suggesting the onset of meiosis (see Larkman, '81). In *A. fragacea* female germ cells there appears to be a process of nuclear maturation in terms of increasing nuclear density and nucleolar development throughout the endodermal phase, which does not appear to be interrupted by mitoses. The larger endodermal germ cells are usually found singly, scattered throughout the gonad, rather than in groups or nests, as might be expected if they were mitotically active. Most of the germ cells accumulate glycogen and some begin synthesis of yolk granules while within the endoderm. It is therefore suggested that, in *A. fragacea*, the germ cells begin meiosis and become oocytes at an early stage while still within the endoderm, before entry into the mesoglea. If mitosis occurs, it is restricted to small cells before the onset of vegetative growth and the accumulation of reserve materials. Schmidt and Schäfer ('80) also indicate that anthozoan female germ cells may become oocytes while within the endoderm and may begin vitellogenesis before entering the mesoglea.

The migration of the female germ cells into the mesoglea was described and clearly illustrated with the light microscope by Spaulding ('74) for the sea anemone *Peachia quinquecapitata*, and has since been mentioned by several authors, including Dunn ('75), Jennison ('79), and Szmant-Froelich et al. ('80). In *A. fragacea* the oocytes appear to move into the mesoglea by a process resembling amoeboid movement, although the endodermal cell bases may also play a part. It is not known whether the oocyte softens the mesoglea around it enzymatically to facilitate entry, but the arrangement of the collagen fibrils immediately in front of the advancing oocyte does not seem to be disturbed or compressed during entry. In *Epiactis*, Dunn ('75) suggested that female germ cells could move from the endoderm into the mesoglea either by amoeboid movement or at cell division. She suggested that during these divisions one daughter cell remained in the endoderm while the other was thrust into the mesoglea, and she showed an hourglass-shaped cell apparently undergoing this process. No evidence of this means of entry was found in *A. fragacea*. Hourglass-shaped cells are commonly observed at the endoderm/mesoglea border, but they do not appear to be undergoing mitosis. Such cells contain a single nucleus surrounded by an intact nuclear envelope. The constriction does not always divide the cell into two equal parts, but can occur at any position. The cell shown entering the mesoglea in *Peachia* by Spaulding ('74) is also constricted in this way, but has an intact nucleus and shows no signs of mitosis. In *A. fragacea* the constriction appears to be produced where the oocyte passes through the endodermal muscle processes, and this might argue that these processes pose more of an obstacle to entry than does the mesoglea itself. It is possible that

the constriction of the oocyte may be exaggerated by the contraction of the gonad and mesentery which occurs on fixation, which might cause the muscle processes to pinch the oocyte more severely.

Schmidt and Schäfer ('80) state that the oocyte nucleus is always situated in the last part of the oocyte to enter the mesoglea. In *A. fragacea*, however, this is not usually the case. In oocytes which enter the mesoglea while still small, up to perhaps 15 μm in diameter, the nucleus is often located anteriorly and enters the mesoglea very early. In larger oocytes the nucleus usually occupies a roughly central position. Only rarely does the nucleus enter very late. At a later stage, after the oocytes have completed entry, the nucleus then migrates to take up a peripheral location close to the trophonema (see below). This movement of the nucleus to one side was also noted for *Epiactis* by Dunn.

Schmidt and Schäfer ('80) describe a vitelline membrane of fine granular material surrounding the surface of anthozoan oocytes, apparently both while within the endoderm and within the mesoglea. For the coral *Astrangia danae*, Szmant-Froelich et al. ('80) describe a vitelline membrane, found only in large oocytes, consisting of a layer of cortical vesicles just beneath the oocyte membrane. The oocytes of *A. fragacea* have no obvious extracellular coat while within the endoderm. As they enter the mesoglea they become covered by a thin layer of very finely granular basal lamina material. From micrographs in which the oocyte is caught in the process of entering the mesoglea, it can be seen that this layer is identical to and continuous with the basal lamina between the epithelial cell bases and the mesoglea, as described for *Anthopleura elegantissima* planulae by Chia and Koss ('79). It appears that as the oocytes enter the mesoglea, they do not break through the basal lamina but rather push it into the mesoglea ahead of them. Once within the mesoglea, the oocyte must presumably produce more basal lamina as it grows. Whether this layer of basal lamina material should be termed a vitelline membane seems doubtful. This layer is not a structure specific to oocytes; most cells in contact with the mesoglea, with the exception of granular amoebocytes (unpublished observation), have such a layer. Groups of developing male germ cells in the mesoglea are surrounded by a similar layer (unpublished observation). Spawned eggs and early embryos of *A. fragacea* are not surrounded by an extracellular layer of any kind (unpublished observation), and neither are the spawned eggs of *Bunodosoma cavernata* (Dewel and Clark, '74). It is therefore suggested that the term vitelline membrane is inappropriate for the extracellular material around mesogleal oocytes.

For some *A. fragacea* oocytes, entry into the mesoglea appears to be facilitated by an outgrowth of the mesoglea around them. Mesogleal outgrowth around oocytes has previously been reported by Dunn ('75) and Schäfer and Schmidt ('80). However, how this outgrowth is brought about is unclear. Singer ('74) investigated collagen synthesis in the sea anemone *Aiptasia diaphana* using electron microscopy and autoradiography and concluded that mesogleal collagen is synthesized by the epithelial cells. Perhaps the oocyte can stimulate the endodermal cells around it to produce mesoglea. Some small oocytes in *A. fragacea*, while within the endoderm, contact the mesoglea by a slender cytoplasmic process (Larkman, '81). These processes might serve to direct mesogleal outgrowth as well as movement of the oocyte into the mesoglea. Alternatively, the same forces acting to drive the oocyte into the mesoglea may result in the mesoglea being pulled out around the oocyte.

Once within the mesoglea, the oocyte loses contact with the endodermal epithelial cells over most of its surface. At one point, however, contact with the endoderm is retained and a specialized structure called the trophonema develops. The trophonema comprises a group of endodermal cells whose bases penetrate the mesoglea and make contact with the surface of the oocyte directly (Larkman and Carter, '82). Trophonemata are found in most Anthozoa (Schmidt and Schäfer, '80), though the details of the structure vary in different species. Trophonemata have recently been observed with the light microscope in a number of anemone species, including *Epiactis prolifera* (Dunn, '75), *Anthopleura elegantissima* (Jennison, '79), *Aulactinia incubans* (Dunn et al., '80), *Condylactis gigantea*, *Phymanthus crucifer* (Jennison, '81), and *Anthopleura handi* (Dunn, '82). A structure resembling a trophonema was shown for *Actinostola spetsbergensis* by Riemann-Zurneck ('76). Carter and Thorp ('79) clearly illustrate a structure which they termed the fertilization pore in *Actinia equina* which appears to correspond to the

trophonema. Many of these authors suggest that the trophonema has a nutritive function, conveying nutrients from the gastrovascular cavity, where extracellular digestion takes place, to the growing oocyte. Some evidence that this is the case in *A. fragacea* was provided by electron microscopy and light microscope autoradiography by Larkman and Carter ('82).

From the present study, it is possible to speculate and suggest a possible mechanism for the formation of trophonema in *A. fragacea*. When oocytes have nearly completed entry into the mesoglea, there appears to be a pause in the entry process. During this period, the small proportion of the oocyte remaining in the endoderm enters into a close relationship with the endodermal cell bases immediately around it. This relationship involves close membrane contact, including intercellular junction formation, between oocyte and endoderm, and interdigitation between endodermal cell bases and oocyte cytospines. As the last portion of the oocyte finally enters the mesoglea, some of the endodermal cell bases move or are dragged with it. Thus the gap in the mesoglea/endoderm border through which the oocyte passed never fully closes, and the oocyte retains contact with the endoderm. The endodermal cells involved then begin specialization to become trophonemal cells.

The proposed scheme is broadly consistent with the findings of Schmidt and Schäfer ('80), although some details of the process in *A. fragacea* differ from their description. In particular, Schmidt and Schäfer state that in the early stages the trophonema consists of cells which have no prominent nucleus and have a fine granular cytoplasm and special electron-dense bodies. In *A. fragacea* the trophonema appears to originate from ordinary gonad epithelial cells, indistinguishable from those around them. They may later become specialized, but they always retain a normal nucleus (unpublished observation). Widersten ('65) reports a sequence of events during scyphozoan oogenesis which bears a striking resemblance to the proposed mechanism of trophonema formation described here. However, he refers to the epithelial cells involved as nurse cells, a term which is usually applied to accessory cells derived from germ cells, which, at least in *A. fragacea*, the trophonemal cells do not appear to be.

During the present study, trophonema initiation was not observed for oocytes which enter the mesoglea at a small size (less than 15 μm), and how the trophonema is formed in these cases is not clear. Nonetheless, Campbell's ('74) suggestion that anthozoan oocytes move into the septal mesoglea at an early stage and appear to mature quite independently with no involvement of somatic cells may now need to be reconsidered.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. M.A. Carter for helpful discussion, encouragement and critical reading of the manuscript, and to his Head of Department, Professor F.A. Hibbert, for the provision of research facilities.

LITERATURE CITED

Anderson, E. (1974) Comparative aspects of the ultrastructure of the female gamete. Int. Rev. Cytol. [Suppl.] *4*:1–70.

Boelsterli, V. (1977) An electron microscopic study of early developmental stages, myogenesis, oogenesis and cnidogenesis in the anthomedusa, *Podocoryne carnea* M. Sars. J. Morphol. *154*:259–290.

Campbell, R.D. (1974) Cnidaria. In A.C. Giese and J.S. Pearse (eds): Reproduction of Marine Invertebrates. Vol. 1. New York: Academic Press, pp. 133–199.

Carter, M.A., and M.E. Funnell (1980) Reproduction and brooding in *Actinia*. In P. Tardent and R. Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 17–22.

Carter, M.A., and C.H. Thorp (1979) The reproduction of *Actinia equina* L. var. *mesembryanthemum*. J. Mar. Biol. Assoc. U.K. *59*:989–1001.

Carter, M.A., and J.P. Thorpe (1981) Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific. J. Mar. Biol. Assoc. U.K. *61*:79–93.

Chapman, D.M. (1974) Cnidarian histology. In L. Muscatine and H.M. Lenhoff (eds): Coelenterate Biology. Reviews and New Perspectives. New York: Academic Press, pp. 2–92.

Chia, F.S., and B.J. Crawford (1973) Some observations on gametogenesis, larval development and substratum selection of the sea pen *Ptilosarcus guerneyi*. Mar. Biol. *23*:73–82.

Chia, F.S., and R. Koss (1979) Fine structural studies of the nervous system and apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*. J. Morphol. *160*:275–298.

Chia, F.S., and M.A. Rostron (1970) Some aspects of the reproductive biology of *Actinia equina* (Cnidaria: Anthozoa). J. Mar. Biol. Assoc. U.K. *50*:253–264.

Chia, F.S., and J.G. Spaulding (1972) Development and juvenile growth of the sea anemone *Tealia crassicornis*. Biol. Bull. *142*:206–218.

Clark, W.H., and W.C. Dewel (1974) The structure of the gonads, gametogenesis and sperm-egg interactions in the Anthozoa. Am. Zool. *14*:494–510.

Dewel, W.C., and W.H. Clark (1974) A fine structural investigation of surface specializations and the cortical reaction in eggs of the cnidarian *Bunodosoma cavernata*. J. Cell Biol. *60*:78–91.

Dunn, D.F. (1975) Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verill, 1869. Biol. Bull. *148*:199–218.

Dunn, D.F. (1982) Sexual reproduction of two intertidal sea anemones (Coelenterata : Actiniaria) in Malaysia. Biotropica *14*:262–271.

Dunn, D.F., F.S. Chia, and R. Levine (1980) Nomenclature of *Aulactinia (=Bunodactis)*, with description of *Aulactinia incubans* n.sp. (Coelenterata : Actiniaria), an internally brooding sea anemone from Puget Sound. Can. J. Zool. *58*:2071–2080.

Eckelbarger, K.J., and J.P. Grassle (1982) Ultrastructure of the ovary and oogenesis in the polychaete *Capitella jonesi* (Hartman, 1959). J. Morphol. *171*:305–320.

Eddy, E.M. (1975) Germ plasm and the differentiation of the germ cell line. Int. Rev. Cytol. *43*:229–280.

Gemmill, J.F. (1920) The development of the sea anemone *Metridium dianthus* (Ellis) and *Adamsia palliata* (Bohad). Philos. Trans. R. Soc. Lond. [Biol.] *209*: 351–375.

Gemmill, J.F. (1921) The development of the sea anemone *Bolocera tuediae* (Johnst.). Q. J. Microsc. Sci. *65*:577–587.

Green, C.R., and P.R. Bergquist (1982) Phylogenetic relationships within the invertebrata in relation to the structure of septate junctions and the development of 'occluding' junctional types. J. Cell Sci. *53*:279–305.

Hyman, L.H. (1940) The Invertebrates: Protozoa through Ctenophora. The Acoelomate Bilateria. New York: McGraw-Hill.

Jennison, B.L. (1979) Gametogenesis and reproductive cycles in the sea anemone *Anthopleura elegantissima* (Brandt, 1835). Can. J. Zool. *57*:403–411.

Jennison, B.L. (1981) Reproduction in three species of sea anemones from Key West, Florida. Can. J. Zool. *59*:1708–1719.

Kessel, R.G. (1968) Electron microscope studies on developing oocytes of a coelenterate medusa with special reference to vitellogenesis. J. Morphol. *126*:211–248.

Larkman, A.U. (1980) Ultrastructural aspects of gametogenesis in *Actinia equina* L. In P. Tardent and R. Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 61–66.

Larkman, A.U. (1981) An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (Cnidaria : Anthozoa). Int. J. Invertbr. Reprod. *4*:147–167.

Larkman, A.U., and M.A. Carter (1982) Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a nutritive function. Int. J. Invertbr. Reprod. *4*:375–379.

Nieuwkoop, P.D., and L.A. Satasurya (1981) Primordial Germ Cells in the Invertebrates. Cambridge: Cambridge University Press.

Noda K., and C. Kanai (1977) An ultrastructural observation on *Pelmatohydra robusta* at sexual and asexual stages, with special reference to 'germinal plasm'. J. Ultrastruct. Res. *61*:284–294.

Noda, K., and C. Kanai (1980) An ultrastructural observation on the embryogenesis of *Pelmatohydra robusta* with special reference to 'germinal dense bodies'. In P. Tardent and R. Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 133–138.

Norrevang, A. (1968) Electron microscopic morphology of oogenesis. Int. Rev. Cytol. *23*:113–186.

Riemann-Zurneck, K. (1976) Reproductive biology, oogenesis and early development in the brood-caring sea anemone *Actinostola spetsbergensis* (Anthozoa : Actiniaria). Helgol. Wiss. Meeresunters *28*:239–249.

Rinkevich, B., and Y. Loya (1979) The reproduction of the Red Sea Coral *Stylophora pistillata*. 1. Gonads and planulae. Mar. Ecol. Prog. Ser. *1*:133–144.

Roosen-Runge, E.C. (1977) The Process of Spermatogenesis in Animals. Cambridge: Cambridge University Press.

Rostron, M.A., and J. Rostron (1978) Fecundity and reproductive ecology of a natural population of *Actinia equina* L. (Cnidaria : Anthozoa). J. Exp. Mar. Biol. Ecol. *33*:251–259.

Schade, M.L., and R.R. Shivers (1980) Structural modulation of the surface and cytoplasm of oocytes during vitellogenesis in the lobster, *Homarus americanus*. An electron microscope-protein tracer study. J. Morphol. *163*:13–26.

Schäfer, W.G., and H. Schmidt (1980) The anthozoan egg: Differentiation of internal oocyte structure. In P. Tardent and R. Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 47–51.

Schmidt, H., and W.G. Schäfer (1980) The anthozoan egg: Trophic mechanisms and oocyte surfaces. In P. Tardent and R. Tardent (eds): Developmental and Cellular Biology of Coelenterates. Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 41–46.

Singer, I.I. (1974) An electron microscopic and autoradiographic study of mesogleal organization and collagen synthesis in the sea anemone *Aiptasia diaphana*. Cell Tissue Res. *149*:537–554.

Spaulding, J.G. (1972) The life cycle of *Peachia quinquecapitata*, an anemone parasitic on medusae during its larval development. Biol. Bull. *143*:440–453.

Spaulding, J.G. (1974) Embryonic and larval development in sea anemones (Anthozoa : Actiniaria). Am. Zool. *14*:511–520.

Stephenson, T.A. (1935) The British Sea Anemones, Vol. II. London: The Ray Society.

Szmant-Froelich, A., P. Yevich, and M.E.Q. Pilson (1980) Gametogenesis and early development of the temperate coral *Astrangia danae* (Anthozoa : Scleractinia). Biol. Bull. *158*:257–269.

Tardent, P. (1974) Gametogenesis in the genus *Hydra*. Am. Zool. *14*:447–456.

Widersten, B. (1965) Genital organs and fertilization in some Scyphozoa. Zool. Bidrag. Uppsala *37*:45–58.

Zihler, J. (1972) Zur Gametogenese und Befruchtungsbiologie von *Hydra*. Wilhelm Roux Arch. *169*:239–267.

Gamete Research 9:303–327 (1984)

An Ultrastructural Study of the Establishement of the Testicular Cysts During Spermatogenesis in the Sea Anemone Actinia fragacea (Cnidaria: Anthozoa)

Alan U. Larkman

Department of Biological Sciences, Portsmouth Polytechnic, Portsmouth, United Kingdom

Spermatogenesis in the sea anemone Actinia fragacea takes place in numerous testicular cysts located in the mesoglea of the gonads. Prospermatogonia arise among the bases of the gonadal epithelial cells bordering the mesoglea, and later migrate into the mesoglea to establish the cysts. The prospermatogonia arise singly, but soon most are found as small groups within the endoderm. They are small cells, 6–7 μm in diameter, and have relatively large nuclei with a single nucleolus. Their cytoplasm is dense, and contains dense bodies and nuage material as well as Golgi, mitochondria, and individual cisternae of endoplasmic reticulum. Each prospermatogonium bears a flagellum, originating in a groove or channel in the cytoplasm.

A small proportion of prospermatogonia enter the mesoglea singly, but most migrate as elongate groups or "slugs" of cells. As they enter, the groups often become constricted into hour-glass shapes, and they become covered by the endodermal basal lamina. During the later stages of entry, the last part of the group to enter retains contact with the bases of the epithelial cells, which are dragged into the mesoglea behind the germ cells. This contact between germ cells and endoderm persists throughout spermatogenesis and prevents closure of the mesoglea behind the group. The endodermal cells involved begin specialization to form the trophonema. Once entry is complete, the groups enlarge rapidly to form the testicular cysts. A small number of germ cells appear to remain behind in the endoderm after most have entered the mesoglea, and the possible significance of these cells is discussed.

Key words: spermatogenesis, Actinia fragacea, ultrastructure, testicular cyst, trophonema

Received August 18, 1983; accepted November 25, 1983.

Address reprint requests to Dr. A.U. Larkman, Department of Biological Sciences, Portsmouth Polytechnic, King Henry 1 Street, Portsmouth, PO1 2DY, United Kingdom.

INTRODUCTION

In sea anemones, spermatogenesis takes place in numerous testicular cysts that are located in the mesogleal layer of the gonad [Dewel and Clark, 1972]. The gonadal mesoglea is covered on each side by a layer of endodermal epithelial cells. The male germ cells arise initially among the basal regions of these epithelial cells, but later they migrate into the mesoglea and establish the testicular cysts.

Early male anthozoan germ cells are small and are difficult to distinguish by light microscopy prior to their entry into the mesoglea. Thus most light microscope studies of anthozoan spermatogenesis give few details of the endodermal phase of germ cell development [Chia and Spaulding, 1972; Chia and Crawford, 1973; Dunn, 1975; Jennison, 1979; Rinkevich and Loya, 1979; Szmant-Froelich et al, 1980]. Chia and Rostron [1970] described gametogenesis in the sea anemone Actinia equina but made no mention of a premesogleal phase. The limited number of electron microscope studies of anthozoan spermatogenesis have tended to deal mainly with mature sperm structure and spermiogenesis rather than the earlier stages [Dewel and Clark, 1972; Clark and Dewel, 1974; Hinsch, 1974; Hinsch and Clark, 1973; Lyke and Robson, 1975; Kleve and Clark, 1976; Schmidt and Zissler, 1979; Larkman and Carter, 1980; Schmidt & Höltken, 1980; West, 1980].

As part of a wider ultrastructural study of gametogenesis in the sea anemone Actinia fragacea, particular attention was paid to the early, less well-studied stages of the process. Descriptions of the early female germ cells have already appeared [Larkman, 1981, 1983]. The present paper describes the early male germ cells while within the endoderm, their entry into the mesoglea, and the establishment of the testicular cysts in which sperm differentiation takes place. Spermiogenesis will be described in a future publication.

Actinia fragacea is an intertidal anemone that was previously thought to be a variety of the species Actinia equina, but which is now thought to be a species in its own right [Carter and Thorpe, 1981].

MATERIALS AND METHODS

Large Actinia fragacea individuals were collected from the intertidal zone of the rocky shore at Wembury, near Plymouth, England, at approximately monthly intervals throughout 1979 and 1980. Pieces of gonad were removed and fixed in 3% glutaraldehyde in a 0.1-M phosphate buffer, pH 7.4, containing 3% sodium chloride. Material for electron microscopy was rinsed in buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated using ethanol, and embedded in EMix epoxy resin. Sections were cut on an LKB Ultratome 111 using glass or diamond knives, stained with uranyl acetate and lead citrate and viewed using a Philips EM 300 electron microscope. Material for light microscopy was embedded in JB4 plastic resin, sectioned at 1–2 μm using an LKB Pyramitome, and stained with toluidine blue.

RESULTS

In the population of A fragacea sampled in this study, the sexes appear to be separate and both show an annual cycle of gametogenic activity, as has previously been suggested by Carter and Funnell [1980]. Spawning usually occurs in summer,

during July and August. The gonads may persist from one breeding season to the next, but, in the male, the next season's germ cells do not appear usually until September, so there is a short period when the male gonads seem to be devoid of germ cells. The germ cells arise among the bases of the gonadal epithelial cells. Initially they occur singly, but by October they are found mainly in small groups, and soon begin to migrate into the mesoglea of the gonad. Some individual cells may enter the mesoglea singly, but most enter as groups or "slugs" of several cells. Once within the mesoglea, the groups of germ cells enlarge rapidly by mitosis and possibly also by fusion with other groups and single cells to form the testicular cysts. By January, the cysts may be 50 μm in diameter, and the cells at the centres of the cysts begin to undergo meiosis and differentiate into spermatocytes, spermatids, and eventually sperm. The major events in the establishment of the testicular cysts are summarized in Figure 1. Throughout this paper, the early male germ cells prior to the completion of entry into the mesoglea are referred to as "prospermatogonia." The following description of the prospermatogonia applies both to single cells and those found in groups, since their appearance and organelle complement are similar.

Structure of Prospermatogonia

The prospermatogonia are usually first seen lying a few micrometres away from the gonadal mesoglea, among the tangled bases of the endodermal epithelial cells (Fig. 2A). They are small cells, usually 6–7 μm in diameter and usually spherical or slightly elongate. They have relatively large nuclei and densely staining cytoplasm.

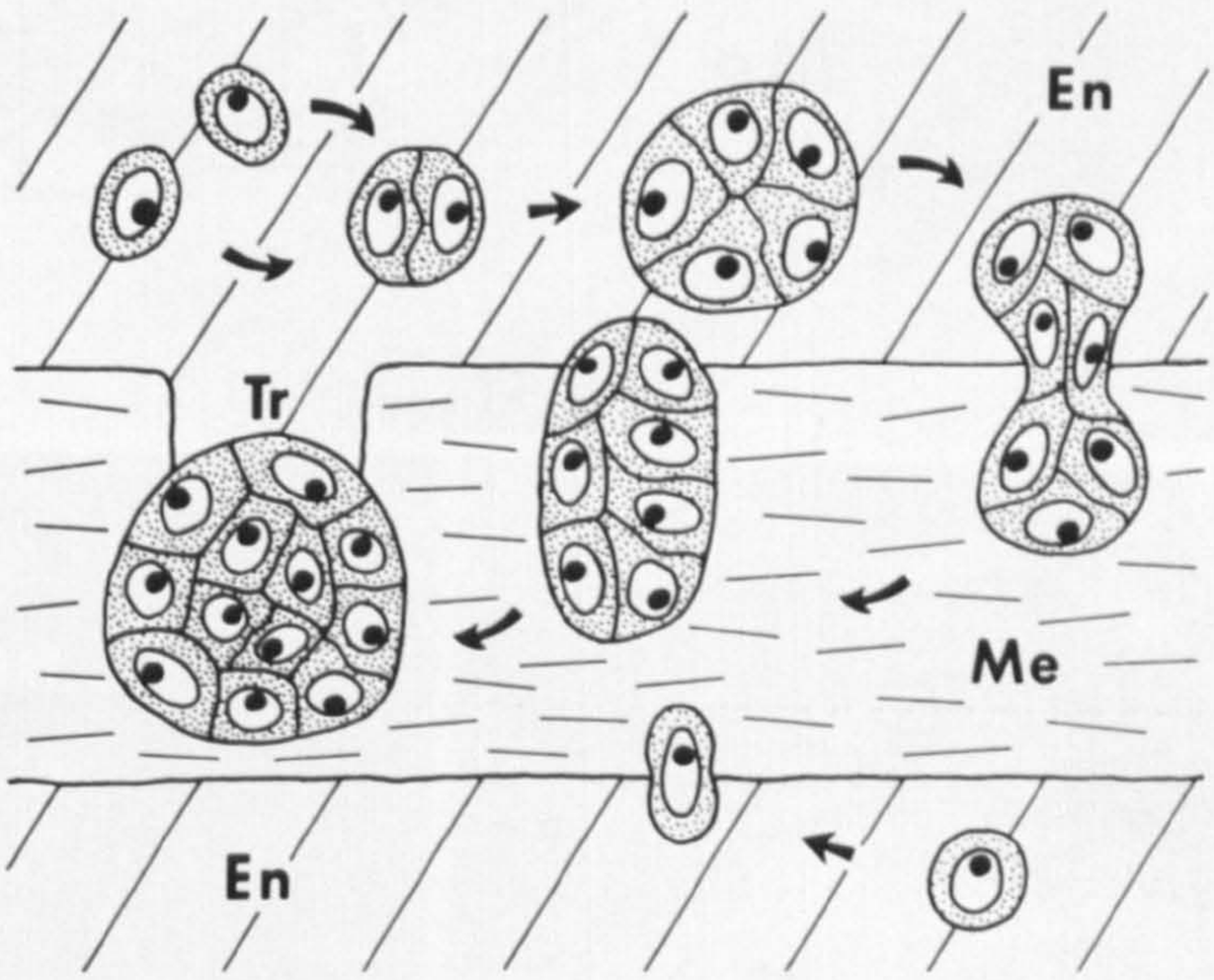
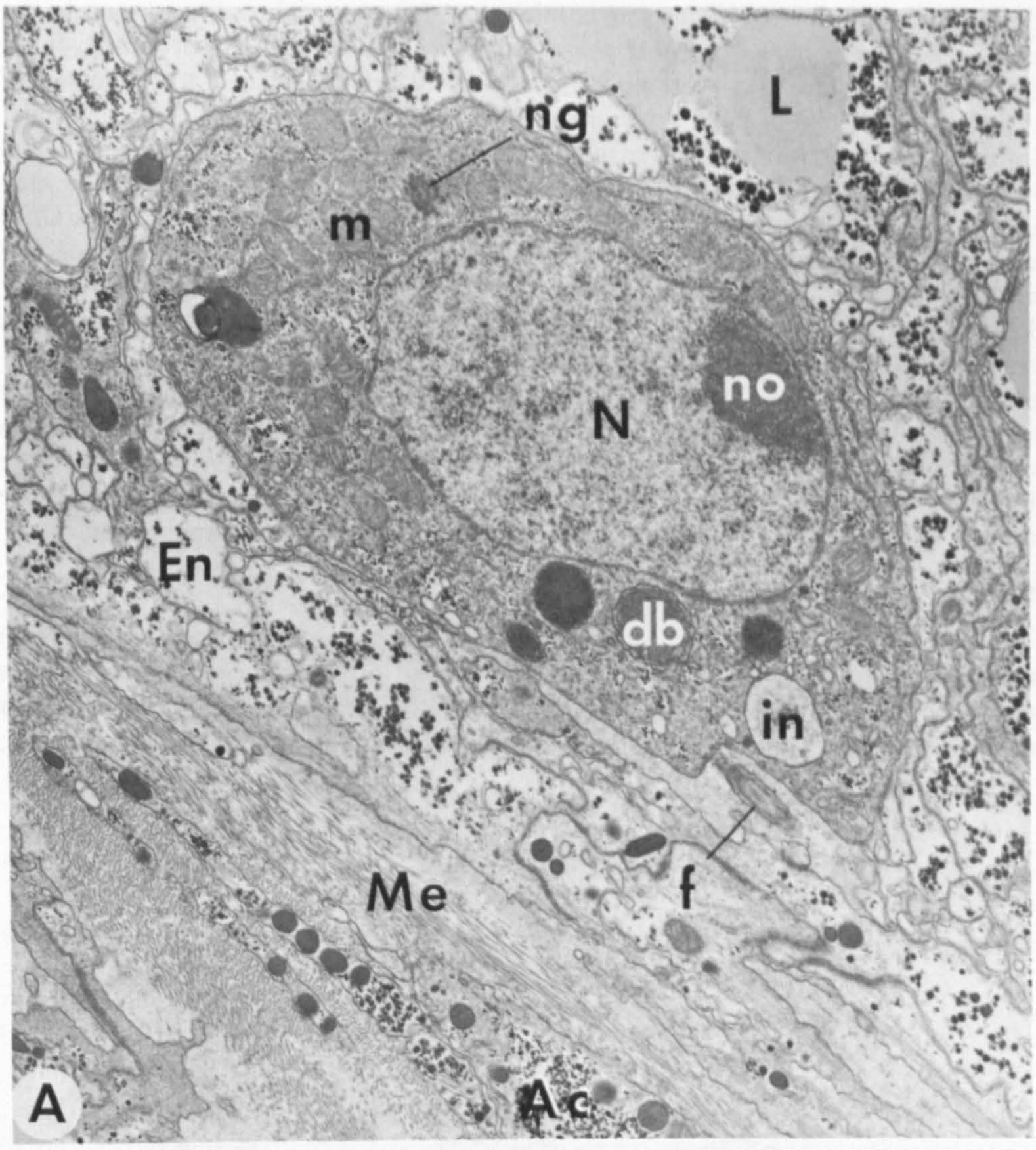
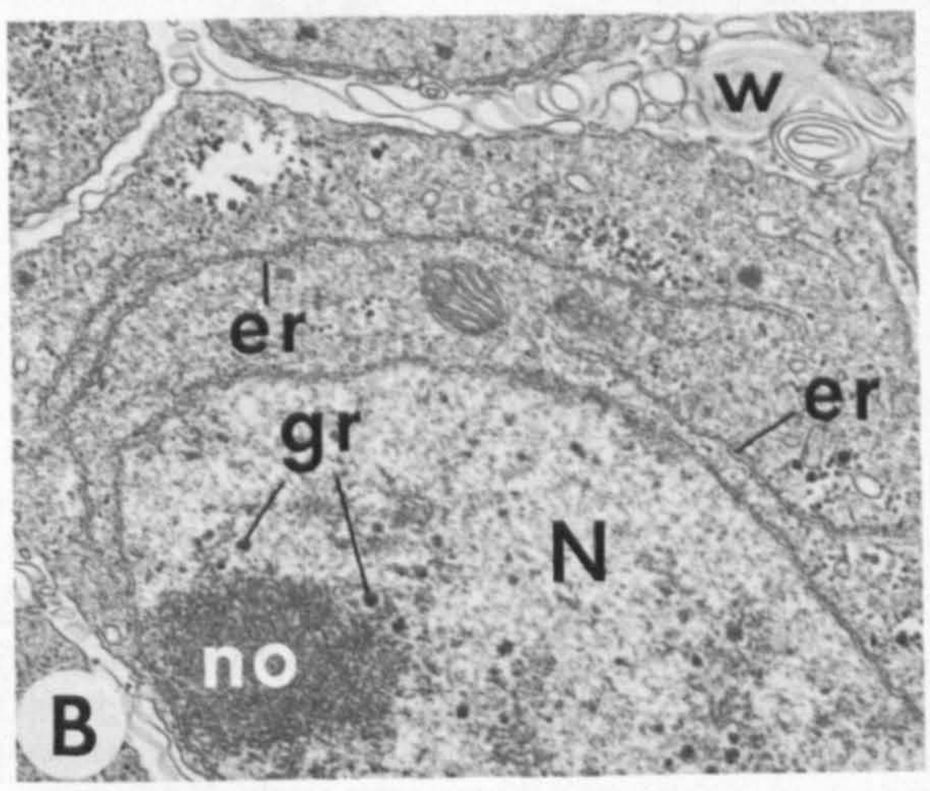
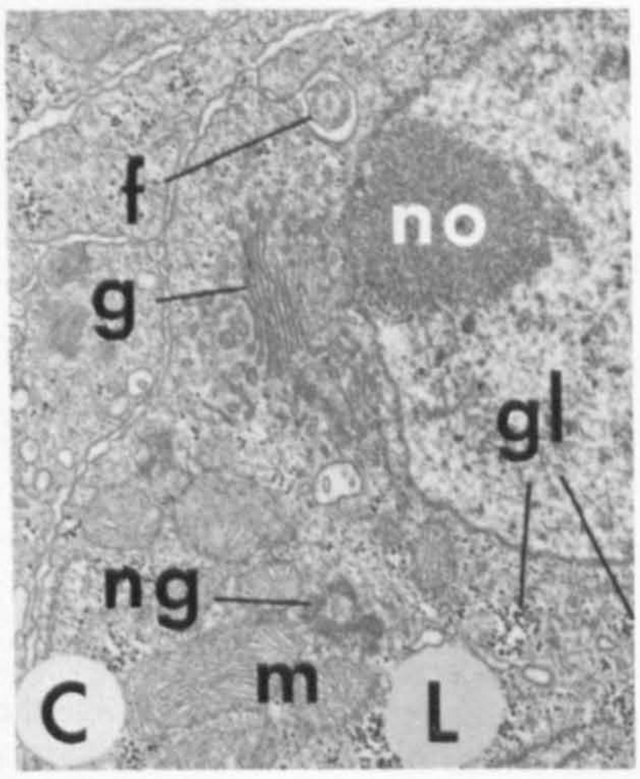


Fig. 1. Summary diagram showing the major steps in the establishment of a testicular cyst. Prospermatogonia arise singly in the endoderm (En, top left), and form groups by aggregation or mitosis or both. These groups then migrate into the mesoglea (Me, centre right) but the last part of the group to enter retains contact with the endoderm and a structure known as the trophonema (Tr) develops (centre left). A small proportion of prospermatogonia (bottom right) enter the mesoglea singly. Not to scale.


ng
L
m
N
no
En
db
in
Me
f
A
Ac


w
er
er
gr
N
no
B


f
no
g
gl
ng
m
C
L

The nucleus is usually 2.5–4 μm in diameter and is of moderate electron density with irregular patches of more dense chromatin material. It also contains numerous small, dense granules, 50–70 nm in diameter (Fig. 2B). There is usually a single nucleolus, which is always located at the periphery of the nucleus, against the nuclear envelope. Rarely are two nucleoli found. The nucleus is surrounded by a typical double membrane nuclear envelope, with occasional nuclear pores. The outer membrane of the envelope is usually studded with ribosomes.

The cytoplasm of the prospermatogonia is generally dense compared with that of the surrounding epithelial cell bases, and contains numerous free ribosomes. The cytoplasm contains a small amount of rough endoplasmic reticulum, which may be present as short lengths, but typically is arranged as individual long cisternae (Fig. 2B) that may extend for several micrometres around the cell, often close to the surface. The cisternae are narrow and contain material of moderate electron density.

The mitochondria usually appear squat and average about 0.3 × 0.6 μm in section, with a dense matrix and numerous narrow, shelflike cristae (Fig. 2C). They are usually clustered into groups rather than randomly scattered through the cytoplasm.

Prospermatogonia are usually seen to contain a single Golgi complex, consisting of a few flattened parallel cisternae (Fig. 2C). The ends of the cisternae may be dilated and contain moderately electron-dense material, while the complex is often surrounded by small, membrane-bound vesicles, 40–80 nm in diameter, containing a similar material.

The cytoplasm contains dense membrane-bound vesicles of various kinds. The largest may be 0.75 μm or more in diameter, are spherical, and contain a highly electron-dense homogenous material (Fig. 2A). Commonly they are rather smaller, less regular in shape and have heterogeneous contents that include membranous material (Fig. 3A,B). These smaller bodies are often found in clusters. Some bodies have loosely fitting membranes, giving them a "haloed" appearance. Some dense bodies contain an angular electron-lucent core (Fig. 3A). Similar bodies have been found in many coelenterate cell types. They may show acid phosphatase activity and are often referred to as residual bodylike inclusions [Chapman, 1974; Tiffon and Hugon, 1977; Larkman and Carter, 1980]. Multivesicular bodies, which are of lower electron density and contain numerous small vesicles, are commonly found (Fig. 3B). Small, apparently empty membrane-bound vesicles are also common (Fig. 3A).

Glycogen is present in all prospermatogonia, usually in the form of numerous small deposits scattered throughout the cytoplasm (Fig. 2C), although larger deposits are occasionally found. Lipid droplets are also common. These may be 1 μm in

---

Fig. 2. A. A prospermatogonium lying close to the mesoglea (Me) among the endodermal cell bases (En), which contain glycogen and lipid droplets (L). The nucleus (N) is relatively large and contains a single nucleolus (no), while the cytoplasm contains several mitochondria (m), dense bodies (db), and nuage material (ng). The cell also has a flagellum (f) lying in a shallow groove, and a cylindrical invagination (in). A process from a granular amoebocyte (Ac) lies within the mesoglea. ×13,500. B. Part of a prospermatogonium within a group. The nucleus (N) contains numerous small granules (gr) as well as a nucleolus (no), and the cytoplasm contains typically extensive cisternae of endoplasmic reticulum (er). The space around the cell contains membranous vesicles and whorls (w). ×20,000. C. The cytoplasm of this prospermatogonium contains a Golgi complex (g), in this case lying close to the nucleolus (no), a lipid droplet (L), mitochondria (M), small glycogen deposits (gl), and nuage material (ng). The flagellum (f) runs in a channel in the cytoplasm, which is almost sealed along its length. ×15,000.

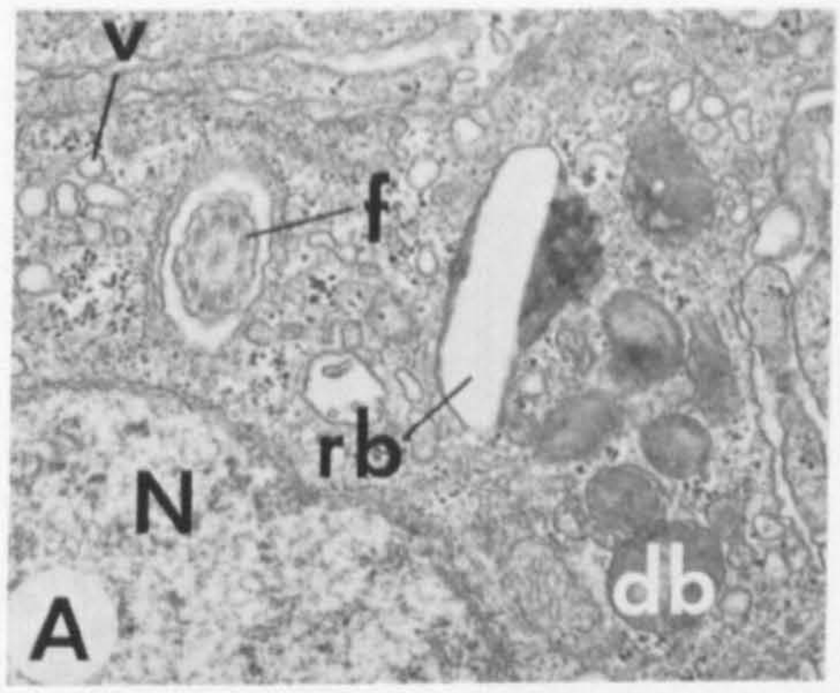
diameter, and often display areas of lower density, which may indicate extraction of components during tissue processing (Figs. 2C, 3C).

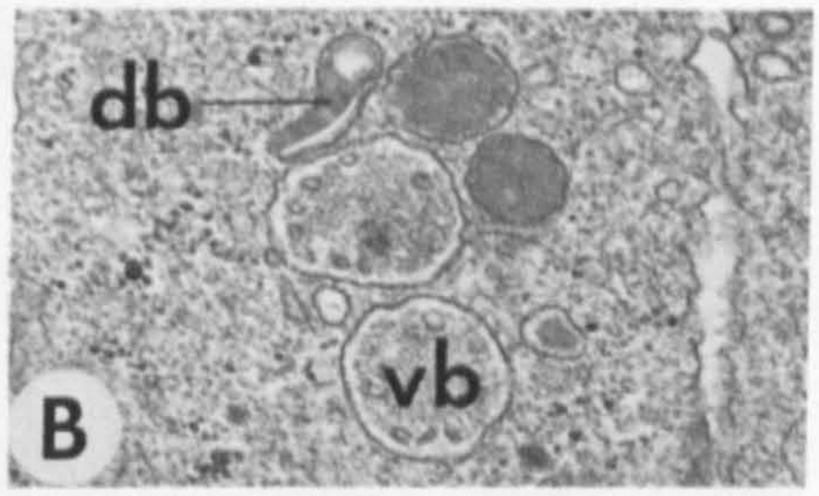
Each prospermatogonium appears to possess a single flagellum. Occasionally the flagellum arises at the surface of the cell, but more commonly it originates some distance from the surface and runs in a groove or channel in the cytoplasm. The groove may be shallow and open (Fig. 2A), deeper and virtually sealed over (Fig. 2C), or, most commonly, it may be a tube, sealed along its length, but open at one end (Figs. 3A,D). It is not clear how far the flagellum projects beyond the surface of the cell. The flagellum originates from a basal apparatus consisting of two centrioles at right angles to each other. Occasionally a third or accessory centriole is seen (Fig. 3E), and the basal apparatus may be seen to include a striated rootlet (Fig. 3F), forming a basal-body-rootlet complex similar to that found in many coelenterate cells [Chapman, 1974]. The flagellum has a typical "9+2" arrangement of microtubules for most of its length. Near its insertion, it shows the usual elaborations that have been described for other coelenterate flagella and sperm tails [Dewel and Clark, 1972; Larkman and Carter, 1980], including a region where Y-shaped fibrils join the microtubular doublets to the flagellar membrane (Fig. 3H). Lengths of flagellar axoneme are frequently found free in the cytoplasm, not enclosed by a plasma membrane (Figs. 3G,H). These cytoplasmic axonemes usually have a precisely organized "9+2" arrangement, but occasionally disrupted patterns are seen (Fig. 3I), the significance of which is not known.

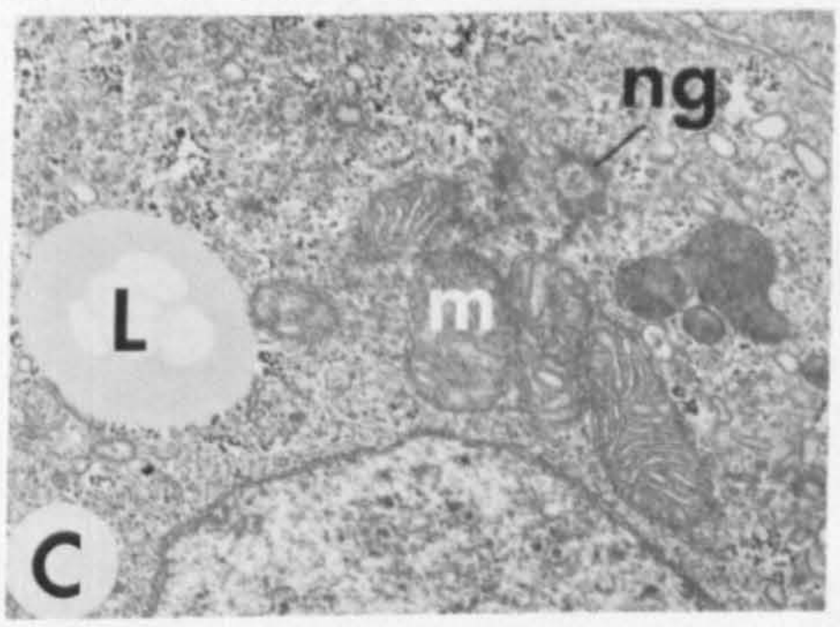
The prospermatogonia contain an unusual dense material that has also been found in female germ cells but not as yet in any nongerminal anemone cell types [Larkman, 1981, 1983], and that has been tentatively termed "nuage material." The nuage is not membrane limited and is most commonly seen as small irregularly shaped areas of finely granular dense material (Fig. 4A). There may be more than one such area per cell. At its most highly organized, and in favourable section, the nuage material may be seen to consist of squat cylinders of dense material, each surrounding a less-dense cylinder of fibrillar material. Several such dense cylinders may be conjoined to form clusters, as in Fig. 4B. Each dense cylinder is about 250 nm in diameter. Often the nuage is intermediate in appearance, with only a suggestion of a ring structure discernible. The nuage material is often found close to, but not in direct contact with, groups of mitochondria (Figs. 2A,C). Another unusual structure has been found in male and female germ cells but not elsewhere as yet. This structure consists of regularly arranged fibrillar hoops closely stacked together (Fig. 4C). In

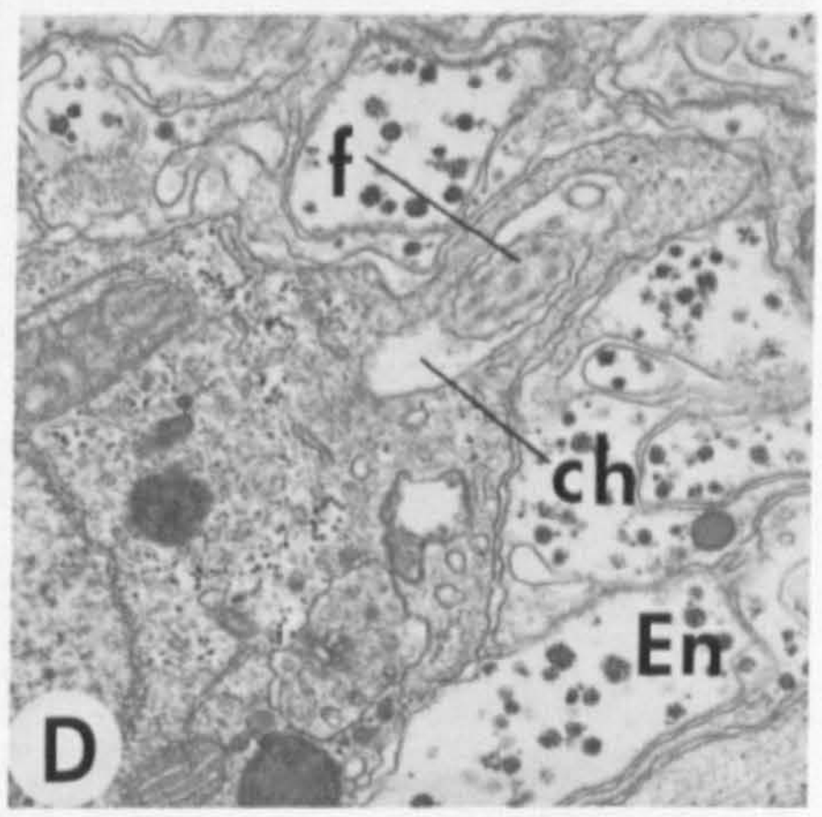
---

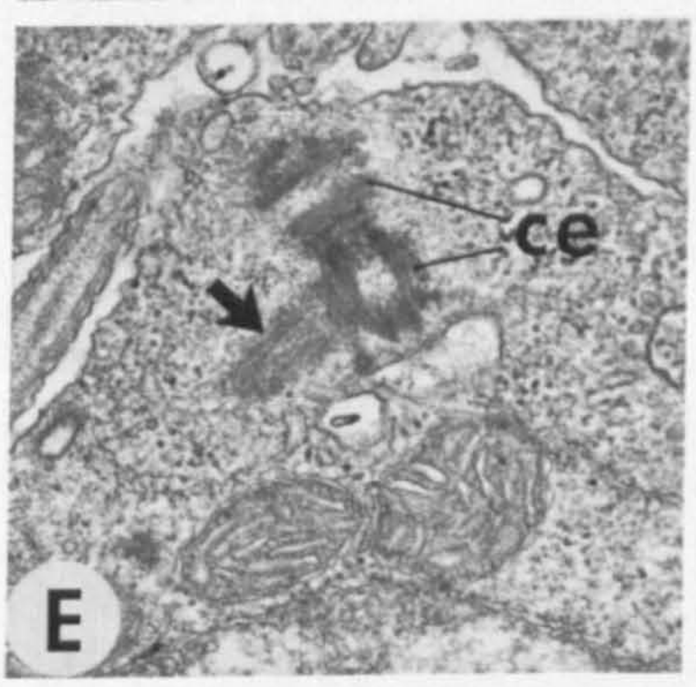
Fig. 3. Cytoplasmic features of prospermatogonia. A. Cytoplasm containing a group of small dense bodies (db), a residual bodylike inclusion (rb) with a characteristic angular electron-lucent core, and small clear vesicles (v). A flagellum (f) lying in a closed cytoplasmic channel can also be seen ×22,000. B. Group of dense (db) and multivesicular bodies (vb). ×22,000. C. Cytoplasm containing a lipid droplet (L) showing signs of extraction, mitochondria (m), and nuage material (ng). ×16,000. D. Prospermatogonium in the endoderm (En) with a flagellum (f) lying in a sealed channel (ch) just beneath the surface of the cell ×21,000. E. Pair of centrioles (ce) lying at right angles to each other, with a third or accessory centriole (arrowed). ×25,000. F. Striated rootlet (sr) as part of the basal apparatus of the flagellum. f, Flagellum. ×25,000. G. Transverse section (TS) through a flagellar axonome (ax) lying free in the cytoplasm ×31,000. H. Longitudinal section (LS) through a cytoplasmic axoneme (ax). The neighbouring cell shows a cylindrical invagination (in) and a flagellum (f) sectioned at the level of the Y-fibrils ×24,000. I. LS through a disrupted cytoplasmic axoneme (dax). ×23,000.

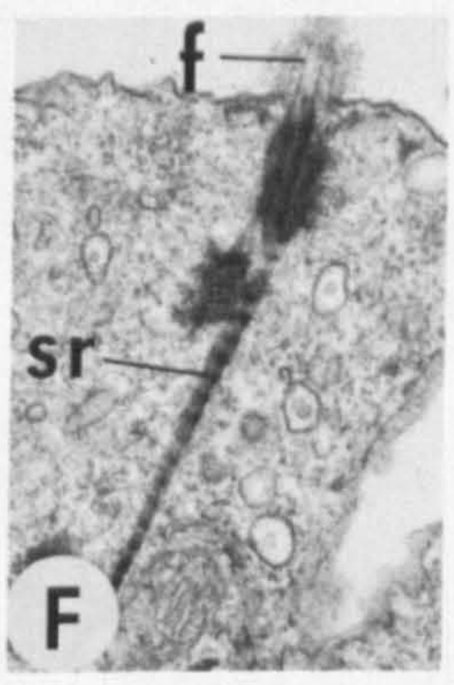

v
f
rb
N
db
A


db
vb
B

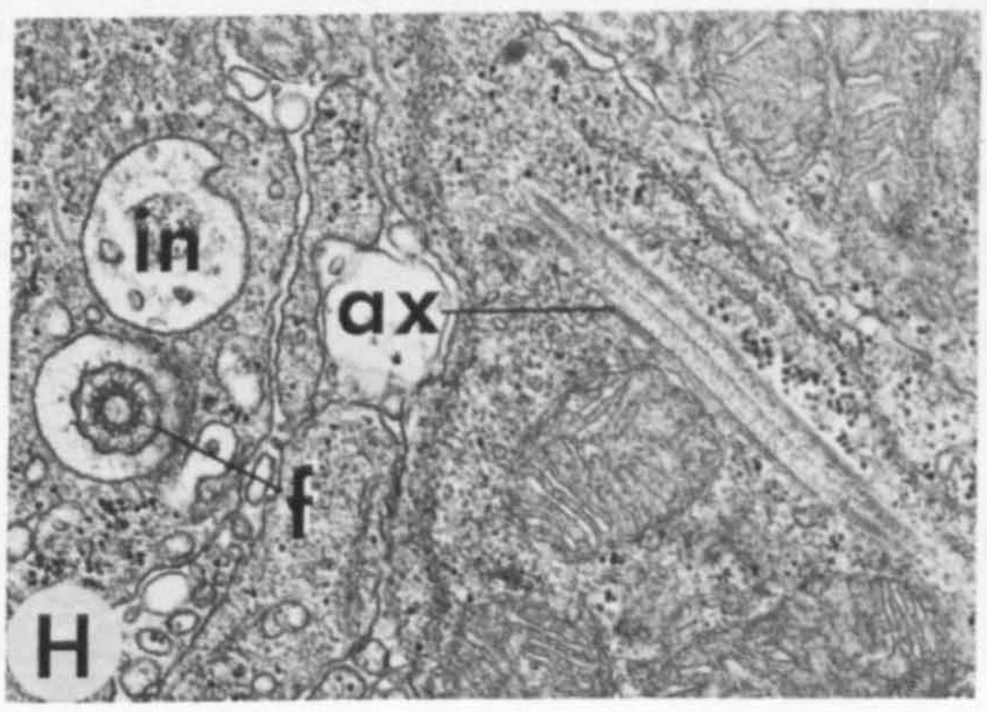

ng
L
m
C

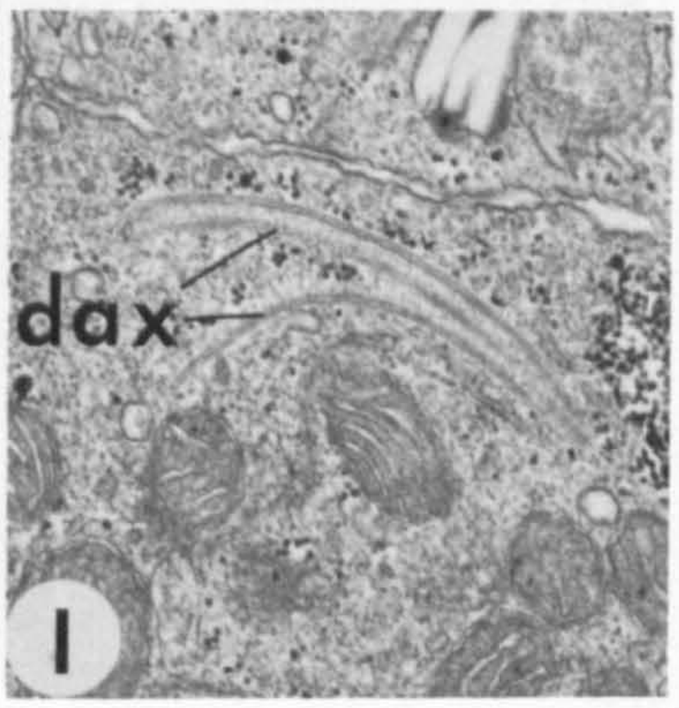

f
ch
En
D

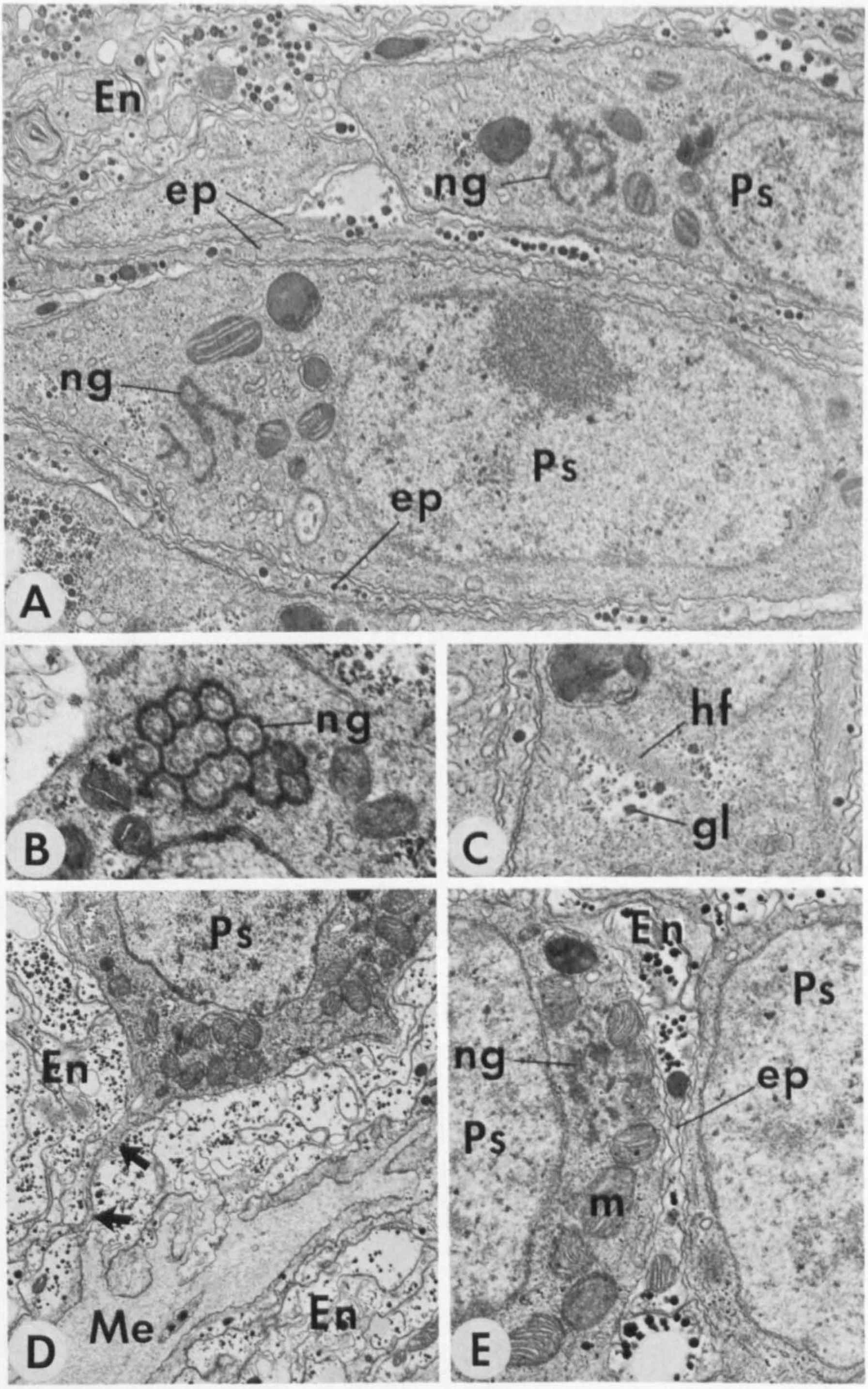

ce
E


f
sr
F


ax
G


in
ax
f
H


dax
I


En
ep
ng
Ps
ng
Ps
ep
A
ng
B
hf
gl
C
Ps
En
Me
En
D
En
Ps
ng
ep
Ps
m
E

both male and female germ cells, these structures are usually closely associated with deposits of glycogen.

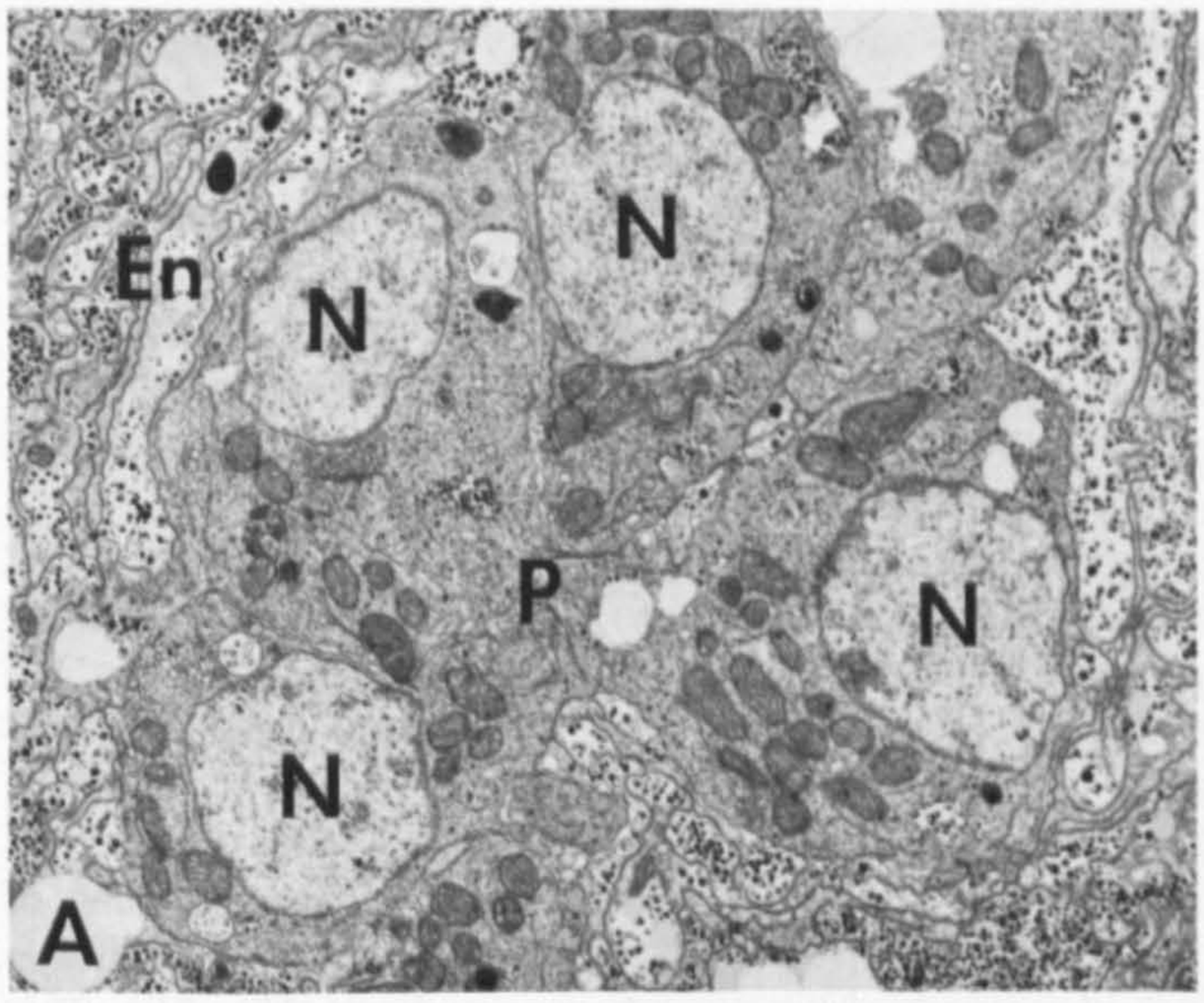
Most of the prospermatogonia found singly in the endoderm are smooth in outline, but some display slender cytoplasmic processes, which may extend toward and make contact with the mesoglea. Occasionally, small side branches extend out from the main gonadal mesogleal layer and may contact individual prospermatogonia. Both situations are seen to occur in Fig. 4D. Some prospermatogonia appear to have a deep, cylindrical invagination of the cell surface that may contain small vesicles and may be lined with a basal laminalike material (Figs. 2A, 3H).

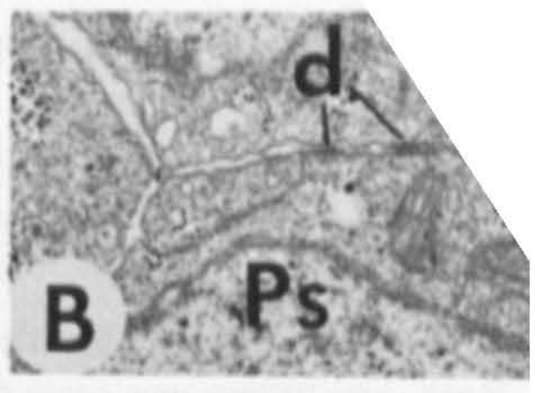
The prospermatogonia appear to arise singly, but by October or November most are found as pairs (Fig. 4E) or small groups (Fig. 5A). Usually the cells comprising these groups are closely packed, being separated from each other by a narrow and apparently empty intermembrane gap. Rarely, desmosomelike intercellular junctions are found between adjacent cells (Fig. 5B). Not uncommonly, however, the cells within a group are separated by slender processes from the surrounding endodermal epithelial cells (Fig. 5C; also 4A,E). These processes often contain bundles of microfilaments (Fig. 4A) or microtubules (Fig. 5C). Occasionally the space between neighbouring cells contains undulating membranous sheets or whorls (Fig. 2B). This is most pronounced around a small minority of cells that appears to have shrunk away from their neighbours (Fig. 5D). In section, these "shrunken cells" appear to have only a very thin rim of cytoplasm surrounding the nucleus.

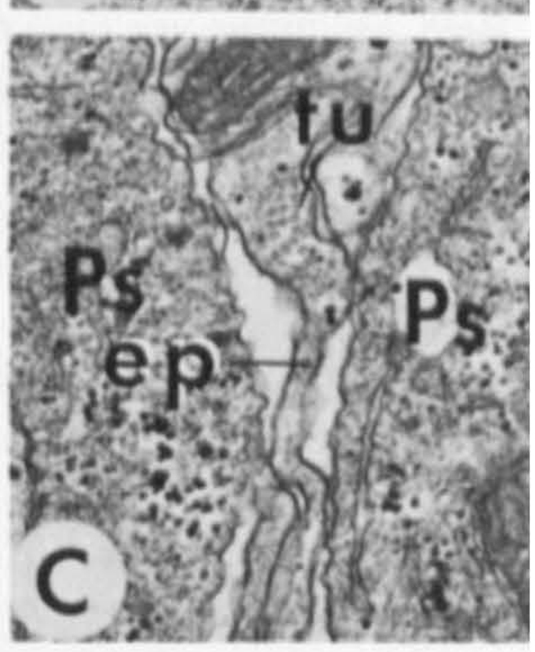
Intercellular bridges have not been observed between the prospermatogonia comprising the groups within the endoderm. In some groups, the cells appear to radiate out from a central focus (Fig. 5A), and often pairs of cells are found separated by a mass of small membranous vesicles (Fig. 5E), but cytoplasmic continuity has never been observed. No instances of cells undergoing mitosis while within endodermal groups have been seen, but this should not be taken as strong evidence that mitosis does not occur (see "Discussion").

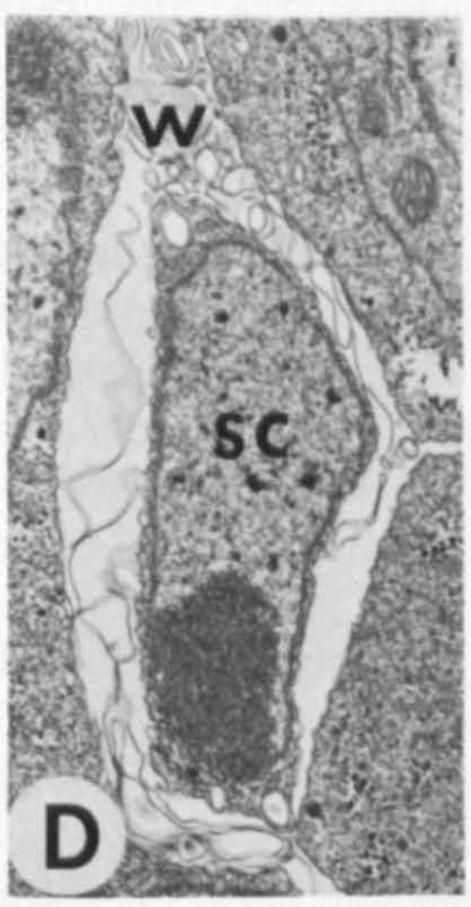
The gonads are situated on the mesenteries, next to the retractor muscles, and there appears to exist a transition zone between the two tissue types. In this region, the epithelial cell muscle processes are more highly developed and the mesoglea is thicker and contains more collagen fibrils that are more regularly packed than is normal in the rest of the gonad. Cells that in many ways resemble germ cells are frequently encountered in this region. These cells tend to be elongate and spindle-shaped rather than rounded, and their mitochondria tend to be small and dense, but otherwise they are very similar to the prospermatogonia described above. They lie in the usual position, a few micrometres back from the mesoglea and the muscle processes (Fig. 5E). Whether their unusual elongate shape is merely imposed by shape changes in the muscular tissue around them, or whether they represent a separate cell population, distinct from the majority of male germ cells, is not known.

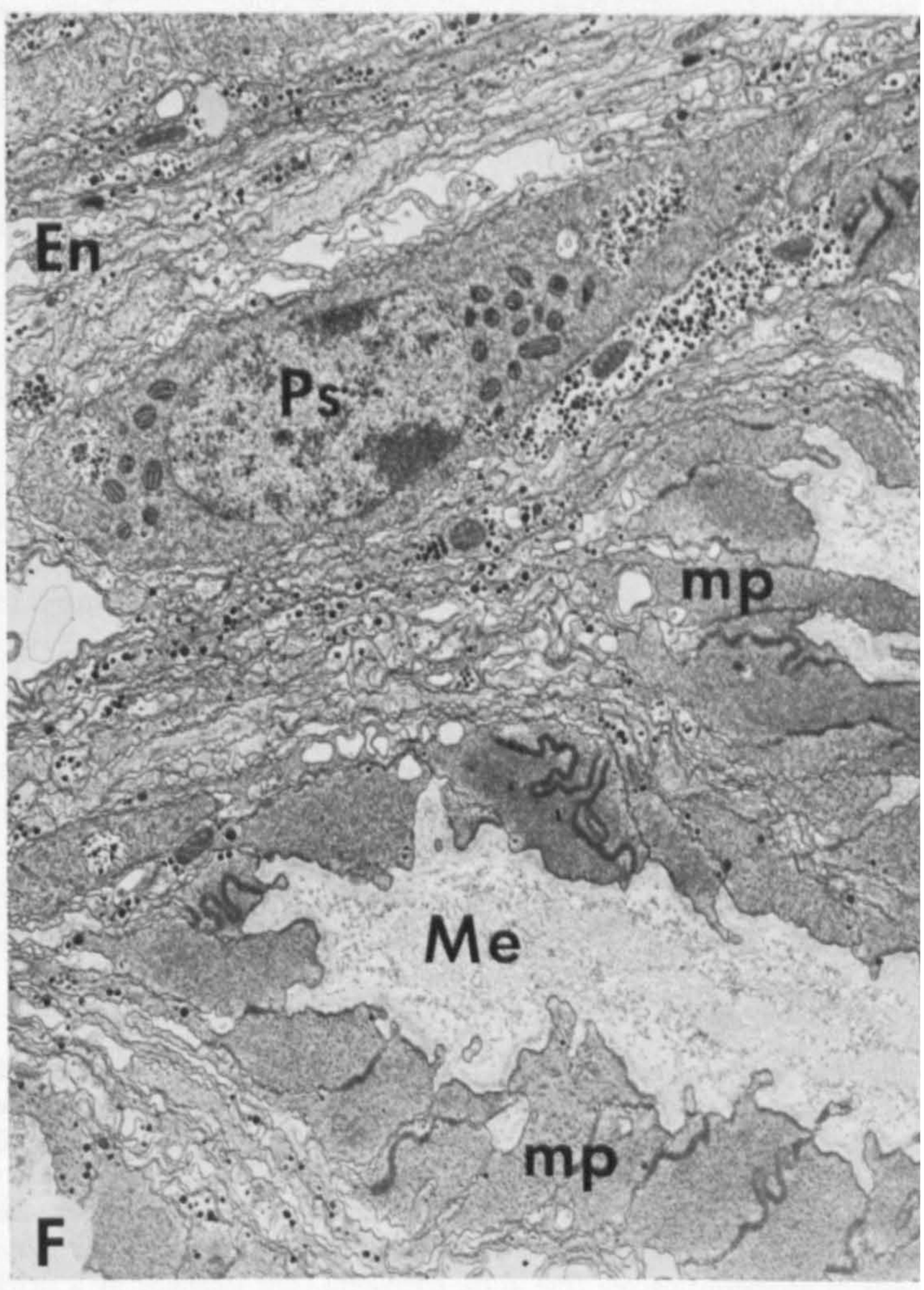
Fig. 4. A. Two prospermatogonia (Ps) lying in the endoderm (En). Both cells clearly show areas of nuage material (ng). The cells are separated by slender processes of the endodermal cells (ep), which contain microfilaments. ×20,000. B. Highly ordered nuage (ng) consisting of conjoined outer dense cylinders surrounding less-dense inner cores. ×27,000. C. Closely stacked fibrillar hoops (hf) associated with glycogen (gl) ×26,000. D. A prospermatogonium (Ps) lying in the endoderm (En) but contacting a side branch of the mesoglea (Me) by means of a slender cytoplasmic process (arrowed). ×8,000. E. A pair of prospermatogonia (Ps) in the endoderm (En) separated by a slender endodermal process (ep). One cell contains nuage material (ng) close to mitochondria (m). × 16,000.


En
N
N
P
N
N
A


d
B
Ps


tu
Ps
Ps
ep
C


w
sc
D


En
Ps
mp
Me
mp
F


Ps
ve
Ps
E

Entry Into the Mesoglea

After a period within the endodermal cell layers, the prospermatogonia migrate into the mesoglea of the gonad. This process was observed in samples taken during October and November. A small proportion of cells may enter the mesoglea singly (Fig. 6A) but by this time most prospermatogonia occur in the endoderm as groups, and these groups enter the mesoglea as "slugs," or elongate clusters of cells. The mechanism of locomotion of both "slugs" and single cells is not clear. The cells show no obvious morphological specializations for movement, but move between the epithelial cell bases by what is presumed to be some form of amoeboid movement. The epithelial cell bases themselves may contribute to the movement of the germ cells. By whatever means, the prospermatogonia separate the bases and muscle processes and make contact with the basal lamina lining the mesoglea. Once contact has been made, mesogleal outgrowth around the germ cells may play an important part in the entry process. In Figure 6B, a group of prospermatogonia lies in contact with the basal lamina, but as yet does not protrude beyond the line of the mesoglea/endoderm boundary. Slender extensions of the mesoglea appear to be growing out around the edges of the group of germ cells.

Soon, however, the group of prospermatogonia does protrude into the mesoglea proper. As the cells pass into the mesoglea, they do not break through the basal lamina but rather push it into the mesoglea ahead of them. Thus the "slug" of prospermatogonia becomes covered by the basal lamina as it enters (Figs. 7A, 8). During entry, the "slug" is usually constricted at the mesoglea/endoderm boundary, giving it a distinctive hourglass shape (Fig. 9A). Some of the cells within such a "slug" become elongate during this process, but whether this shape change is active or passive is not known. The leading part of the group expands within the mesoglea as more cells enter, finally producing a roughly spherical group of cells within the mesoglea after entry is complete.

It appears that the last part of the prospermatogonial group to enter the mesoglea retains contact with the endodermal cell bases adjacent to it, and these bases are dragged into the mesoglea behind the germ cells. This produces a "plug" of endodermal bases between the mesogleal outgrowths around the prospermatogonia (Fig. 7B). This "plug" prevents overgrowth of this region by the mesoglea, and the contact between germ cells and endoderm is maintained throughout spermatogenesis. It also means that the basal lamina around the germ cells remains continuous with that of the gonadal epithelium. Close membrane contact between endoderm and germ cells occurs in this region, although no elaborations such as intercellular junctions have been observed forming between the two. The endodermal cell bases are often rich in glycogen and lipid droplets, and may contain bundles of microfilaments. At a later

---

Fig. 5. A. A group of prospermatogonia within the endoderm (En). The nuclei (N) are located peripherally, and the cells appear to radiate from a central point (P). These cells are not separated by endodermal processes ×6,500. B. Desmosomes (d) linking neighbouring prospermatogonia (Ps) within a group. ×13,000. C. Prospermatogonia (Ps) in a group separated by an endodermal process (ep) containing microtobules (tu). ×28,000. D. Wavy membranous sheets and whorls (w) surrounding a "shrunken cell" (sc). ×12,000. E. Mass of small vesicles (ve) between a pair of prospermatogonia (Ps). ×20,000. F. Spindle-shaped prospermatogonium (Ps) in the endoderm (En) of the gonad near the retractor muscle, where the muscle processes (mp) are highly developed. ×9,000.

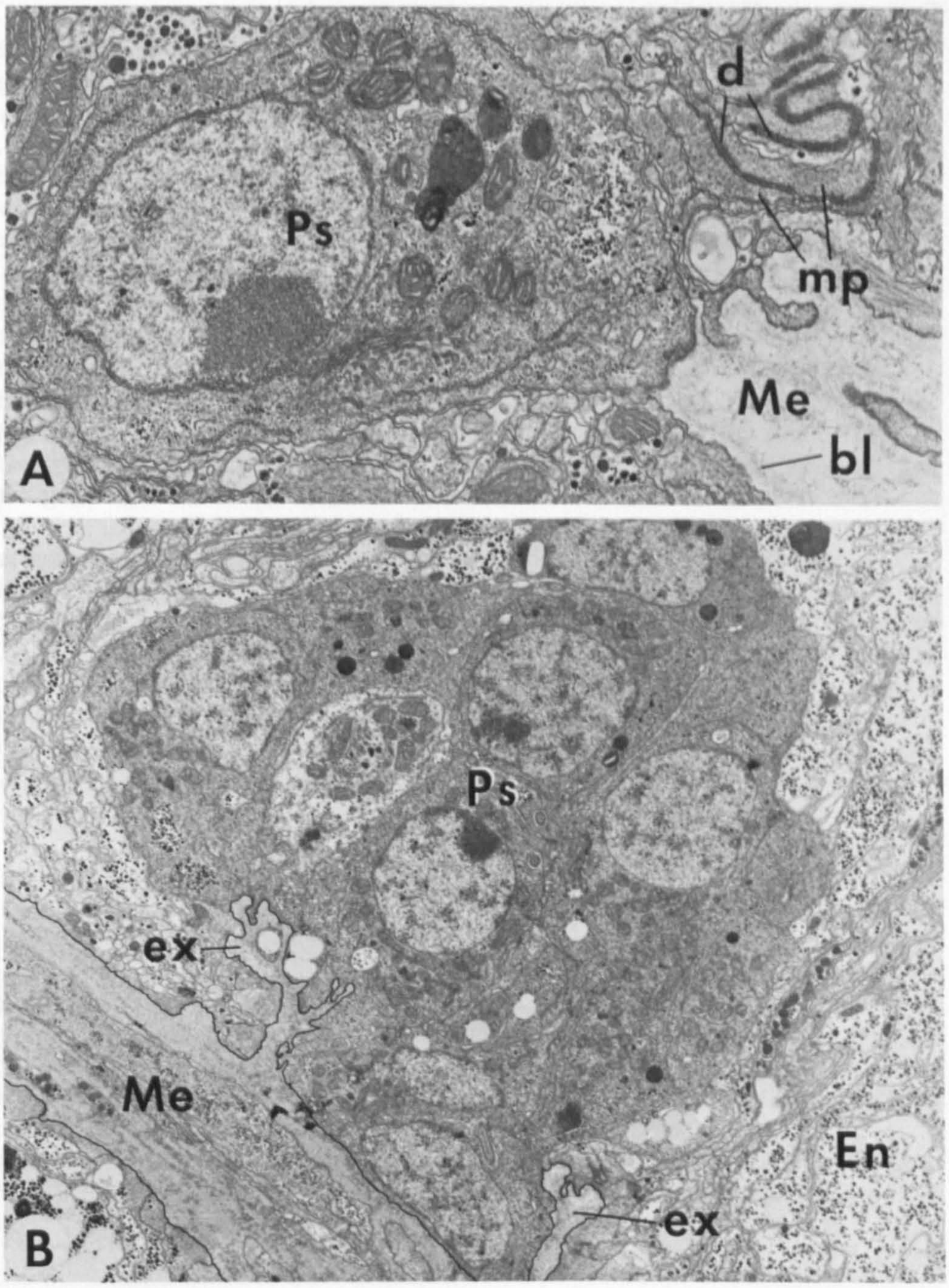


Fig. 6. A. A single prospermatogonium (Ps) beginning entry into the mesoglea (Me). The cell has parted the muscle processes (mp) and contacted the basal lamina (bl). The extensive septate desmosomes (d) joining some muscle processes can be clearly seen ×16,000. B. A group of prospermatogonia (Ps) within the endoderm (En) but touching the mesoglea (Me). The boundary of the mesoglea has been outlined in ink for emphasis. Extensions from the mesoglea (ex) appear to be growing out around the group of germ cells. ×5,000.

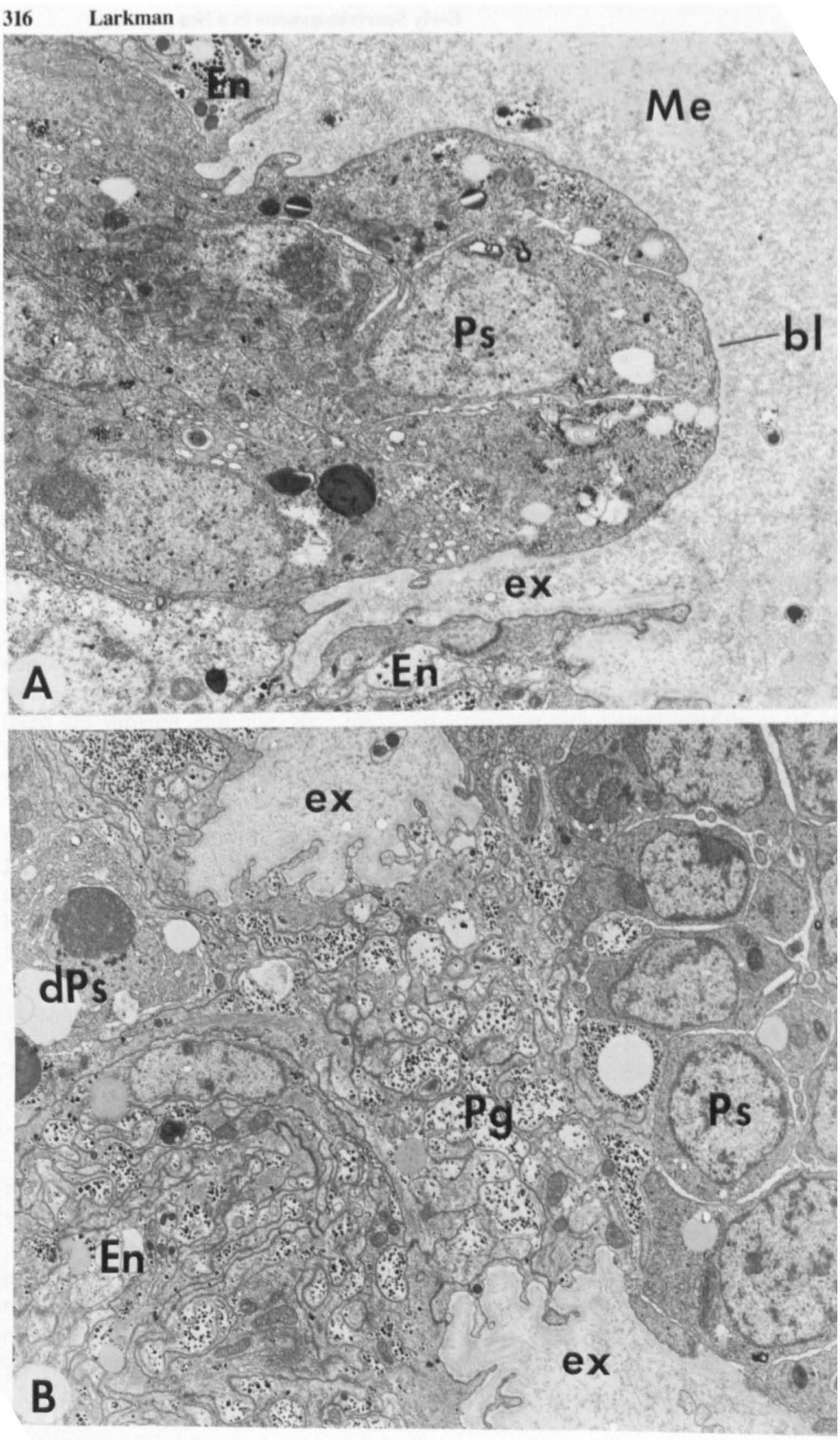
stage, the endodermal cells in contact with the germ cells begin to specialize and give rise to a structure that, by analogy with the female gonad, may be termed a trophonema (see "Discussion"). The nuclei of the endodermal cells are normally located apically, but after the initiation of the trophonema, endodermal nuclei are often found close to the region of contact with the germ cells (Fig. 9B). The major features of this stage are shown in Figure 10.

During and soon after entry into the mesoglea, many groups of prospermatogonia contain usually a single cell of rather different appearance. These cells are the same size and shape as the germ cells, but are of much lower electron density, and hence have been termed "light cells' (Fig. 11A). Their nuclei are surrounded by an intact nuclear envelope but contain only a faint, finely fibrillar material. Their cytoplasm also appears sparse and poorly organized (Fig. 11B). It may contain normal-looking mitochondria, but other organelles are either disrupted or absent. The cell membrane may also appear discontinuous. The cytoplasm may contain axonemal microtubules and structures resembling nuage material, so it seems likely that these cells orginate from germ cells, although they now appear to be degenerate. Occasionally, one or more prospermatogonia may appear to become separated from a group and be "left behind" in the endoderm as the group enters the mesoglea. These cells appear to degenerate rapidly (Fig. 7B), but at no stage come to resemble "light cells."

As the groups of germ cells enter the mesoglea, they very often become associated with granular amoebocytes. The amoebocytes become flattened around the groups of prospermatogonia and are always positioned in a similar way, which suggests that this association is not merely fortuitous. They come to lie closely against the layer of basal lamina that surrounds the germ cells, and thus separate the basal lamina from the fibrous mesoglea (Fig. 11C). A variable proportion of the surface of the prospermatogonial group may become covered in this way, but amoebocytes never form a continuous layer around the germ cells.

Once entry into the mesoglea is complete, the groups of germ cells enlarge to form the testicular cysts in which sperm differentiation takes place. The original gonad mesoglea was only a few micrometres thick, and the testicular cysts may reach a diameter of 150 μm at maturity, so the mesoglea and epithelial sheets of the gonad are deformed as the cysts enlarge. The layer of mesoglea around the cysts becomes thinner and less well endowed with collagen fibrils as the cysts grow (Figs. 11D,E, 12A). Cells undergoing mitosis are seen occasionally, although not frequently, in early growing cysts (Fig. 11E). In these cells, the mitochondria and cisternae of endoplasmic reticulum may become ringed around the nuclear areas as mitosis proceeds. The cysts may reach a considerable size, perhaps 50 μm in diameter (Fig. 12A), before cells at the centre of the cysts begin to differentiate to become spermatocytes and begin meiosis.

During January and February, at a time when most prospermatogonia have left the endoderm and entered the mesoglea, cells resembling germ cells are occasionally found in the endoderm. These cells are often found at the periphery of the gonad, especially near its junction with the retractor muscle. These cells may be larger than most prospermatogonia, reaching 10 μm in diameter. Their cytoplasm may be more extensive and contain greater quantities of glycogen and lipid (Fig. 12B). It may also contain nuage material and hooped fibrillar structures. Thus these cells closely resemble prospermatogonia or perhaps oocytes of similar size (see "Discussion").


En
Me
Ps
bl
ex
A
En
ex
dPs
Pg
Ps
En
ex
B

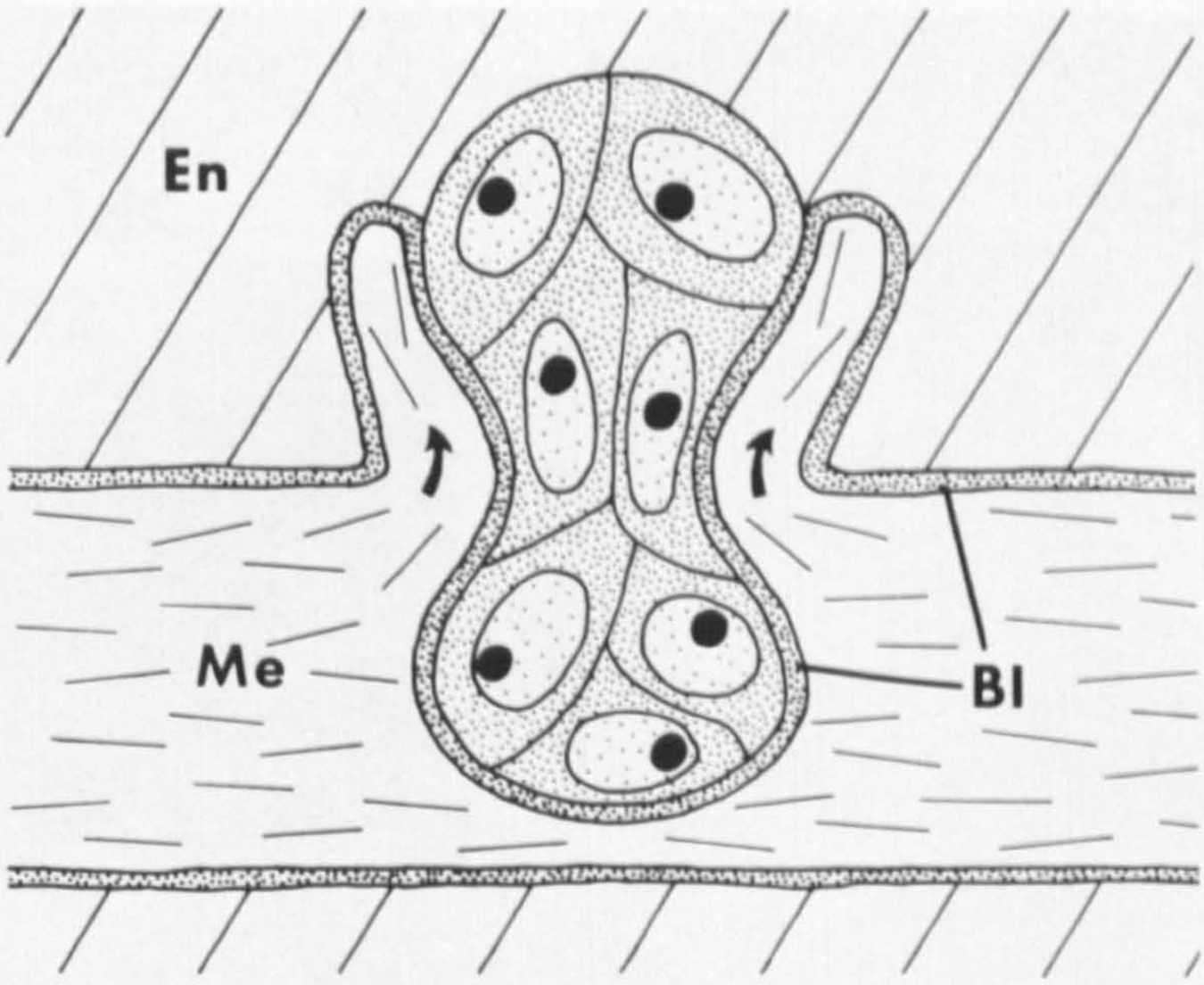
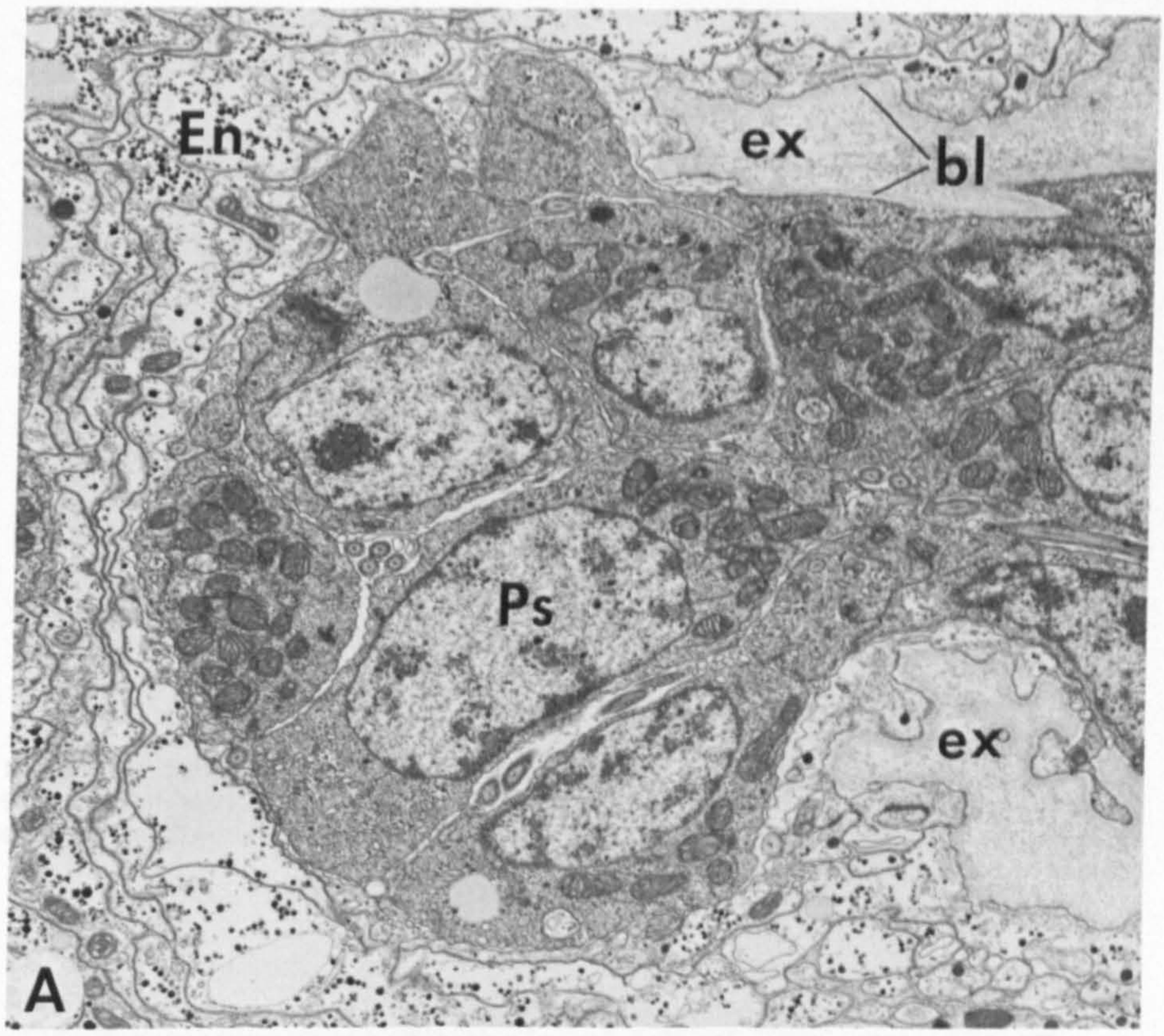


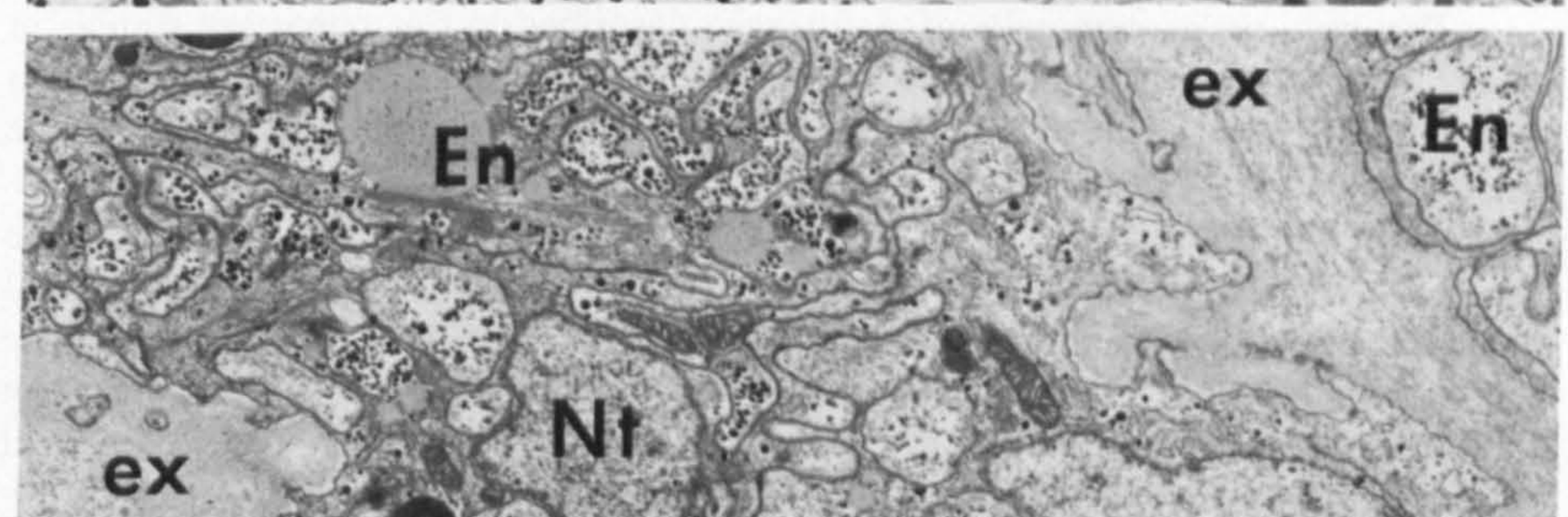
Fig. 8. Diagram illustrating the main features of entry into the mesoglea. The group or "slug" of prospermatogonia becomes constricted into an hourglass shape as it passes from endoderm (En) into the mesoglea (Me). Mesogleal outgrowth around the entering group usually occurs (arrows), and the group becomes covered by the basal lamina (Bl) during entry. Not to scale.

DISCUSSION

The early male germ cells of A fragacea described in this paper are similar in appearance, although not in behaviour, to the early female germ cells described in previous papers [Larkman, 1981, 1983]. Both types of cell morphologically resemble the interstitial cells found in Hydrozoa. These have been extensively studied, especially in Hydra [for a review see Bode and David, 1978] and which are known to give rise to the gametes. However, the origin of anthozoan germ cells is less certain, and the present study has not provided evidence to confirm previous light microscope reports [Dunn, 1975; Jennison, 1979; Szmant-Froelich et al, 1980] that anthozoan germ cells also derive from interstitial cells. In A fragacea, the prospermatogonia seem to arise, evenly distributed throughout the gonad, in early autumn. No population of interstitial cells could be identified within the gonad prior to that time. It is possible that the germ cells could arise at an extragonadal site and migrate into the gonad, but no evidence of this was found. At present, the origin of the germ cells in A fragacea remains unclear, and in the absence of firm evidence, it is perhaps unwise to assume that they derive from interstitial cells.

Fig. 7. A. The leading edge of a group of prospermatogonia (Ps) migrating from the endoderm (En) into the mesoglea (Me). The group becomes covered by the basal lamina (bl) as it enters, and an extension of the mesoglea (ex) is growing out around it. ×10,000. B. The "plug" (Pg) of epithelial cell bases that have been dragged out of the endoderm (En) between the mesogleal extensions (ex) by the advancing group of prospermatogonia (Ps). A solitary prospermatogonium (dPs) appears to have been left behind in the endoderm and is now degenerating. ×6,000.


En
ex
bl
Ps
ex
A


ex
En
En
Nt
ex
Nt
Ps
B

Schmidt and Höltken [1980], speaking of the Anthozoa as a whole, state that the early male cells differ from early oogenic cells only by having a prominent centriolar complex. In A fragacea, however, this distinction would not appear to hold true, since the early female cells may have flagella and centriolar complexes identical to those found in the male [Larkman, 1981, 1983]. The present study has revealed minor differences between early male and female germ cells, although they are undoubtedly very similar. In the population of anemones studied here, the early female germ cells first appear in the endoderm in late spring and enter the mesoglea throughout the summer months. Prospermatogonia, on the other hand, first appear in September or October, and generally have entered the mesoglea by December. The prospermatogonia tend to have more dense nuclei, with better-defined nucleoli than do female germ cells of comparable size, and their cytoplasm contains more dense bodies and multivesicular bodies than in the female. The single long cisternae of endoplasmic reticulum would also seem to be a predominantly male chracteristic, and the male cells usually contain less glycogen and lipid than female cells of the same size. However, these differences are not marked, and may be obscured by the considerable variation in appearance shown by different cells even from the same animal.

In A fragacea, flagella and associated centriolar structures were found even in the very earliest male germ cells observed. Flagella were found among groups of prospermatogonia in the endoderm and entering the mesoglea that did not appear to contain any later stage cells. Dewel and Clark [1972] found flagella and associated structures in spermatocytes of the sea anemone Bunodosoma cavernata and remarked that it was interesting that they should occur in prespermatid stages. Hanisch [1972] found that in the hydrozoan Eudendrium racemosum flagella first appear at the primary spermatocyte stage. Schmidt and Höltken [1980] found flagella in early anthozoan male cells, and raised the possibility that this finding might be evidence that the germ cells had arisen from flagellated endodermal epithelial cells. While no evidence that this was the case was obtained in the present study it remains an interesting suggestion.

Schmidt and Höltken [1980] also found flagellar axonemes lying within the cytoplasm of anthozoan germ cells, as was found in A fragacea. They suggest that they might represent a stage in the formation of the typical external flagellum. Such cytoplasmic axonemes were not found in early female germ cell in A fragacea [Larkman, 1981] and have not been reported from females of other anemone species.

Nuage material has been found in the germ cells of many animal species, and is generally thought to be the equivalent of the germ plasm of amphibians and insects. As such, it may act as a cytoplasmic germ cell determinant [Eddy, 1975; Nieuwkoop and Satasurya, 1981]. Nuage material has been reported from the spermatogonia of the hydrozoan Phialidium gregarium [Roosen-Runge and Szollosi, 1965; Roosen-

Fig. 9. A. The posterior region of a group of prospermatogonia (Ps) in the process of entering the mesoglea. The group is constricted into an hourglass shape where it passes between the mesogleal extensions (ex). The basal lamina (bl) around the group is continuous with that covering the rest of the endoderm (En). No endodermal nuclei are found close to the germ cells at this stage. ×7,000. B. The "plug" of endoderm (En) between the mesogleal extensions (ex) behind a group of prospermatogonia (Ps) that has now completed entry. Nuclei of what may now be considered trophonemal cells (Nt) lie close to the germ cells. ×7,000.

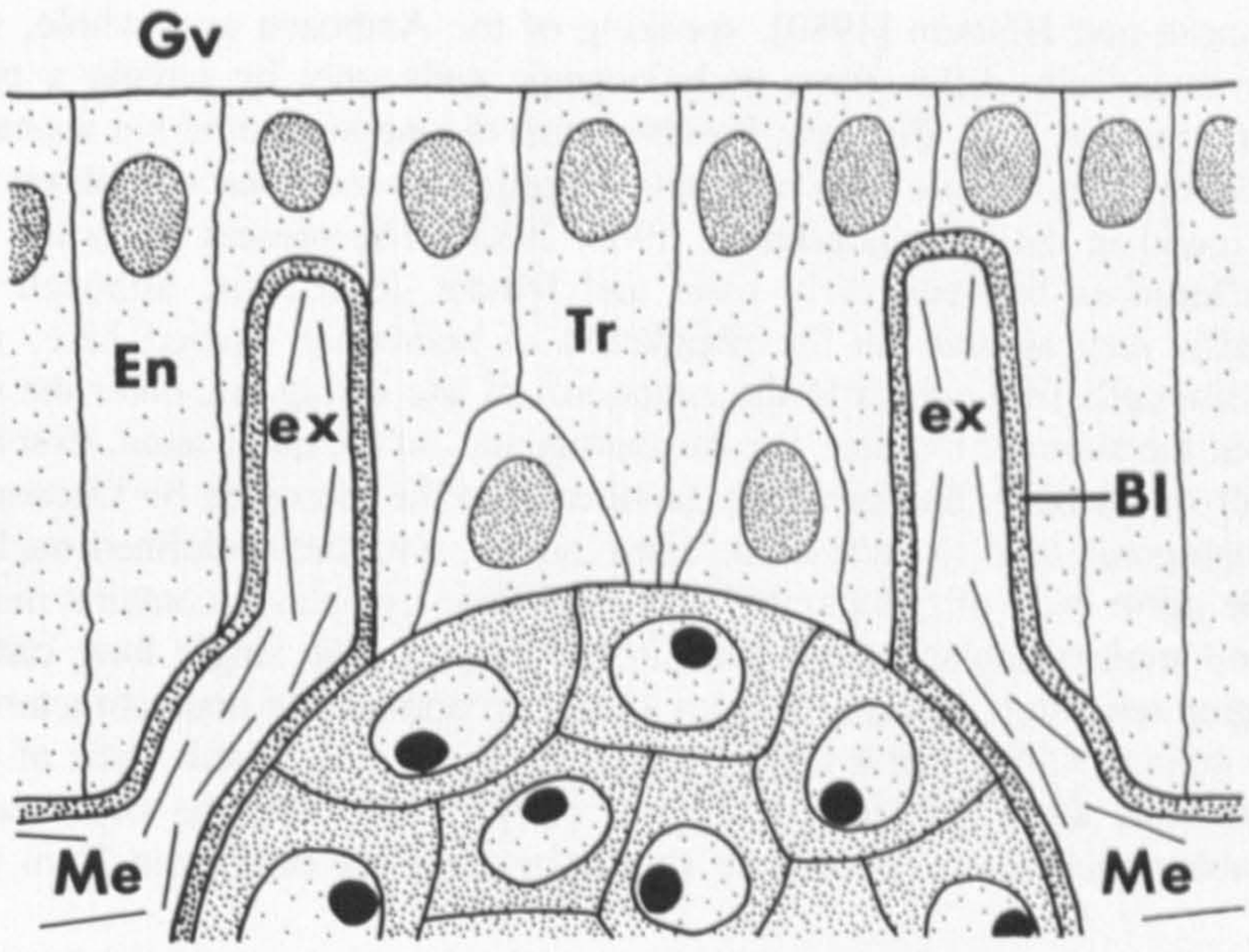
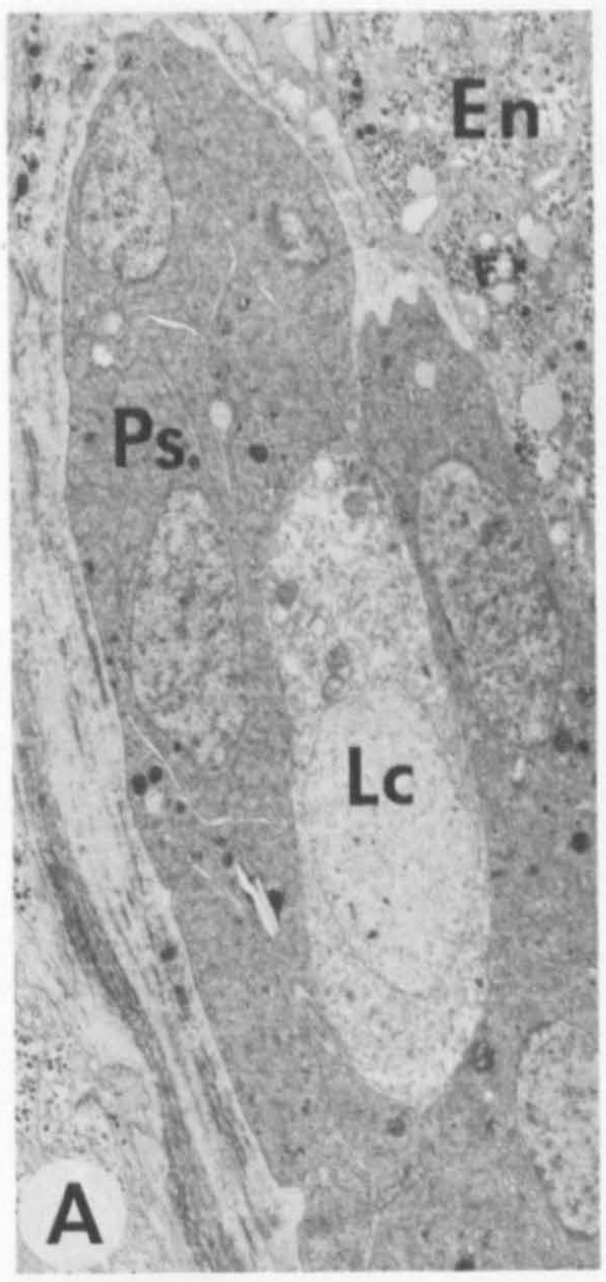


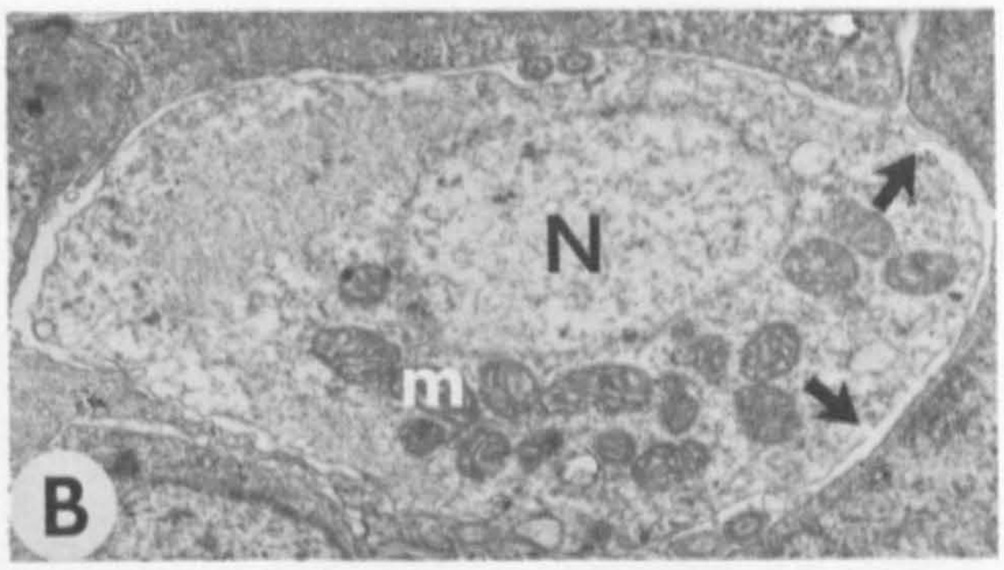
Fig. 10. Diagram illustrating the relationship between the spermatogonia (bottom) and the endoderm (En) after entry into the mesoglea (Me). The endodermal cells between the extensions of the mesoglea (ex) remain in contact with the spermatogonia and begin to specialize to form the trophonema (Tr). Some of these cells appear to depart from the normal epithelial arrangement and have their nuclei close to the interface with the germ cells. Gv, gastrovascular cavity; Bl, basal lamina. Not to scale.

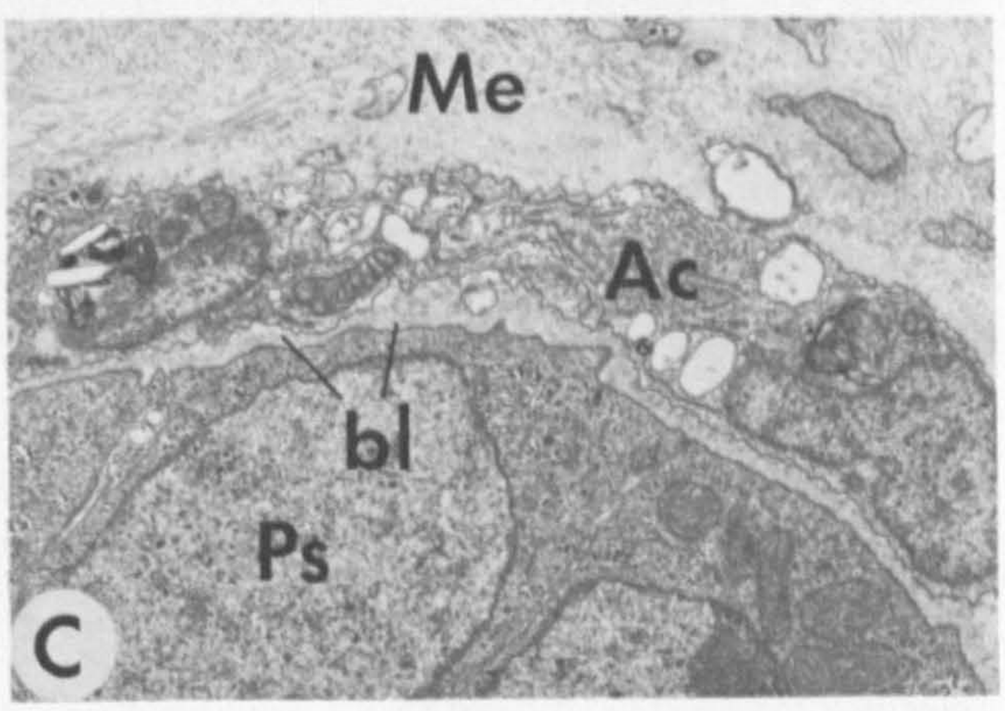
Runge, 1977], and resembles the nuage described here from A fragacea. Until more is known about the origin of the germ cells in sea anemones, it is difficult even to speculate on the possible functional significance of nuage in these animals. However, the consistent finding of such material in both male and female germ cells in this species [Larkman, 1981, 1983] may prove to be significant.

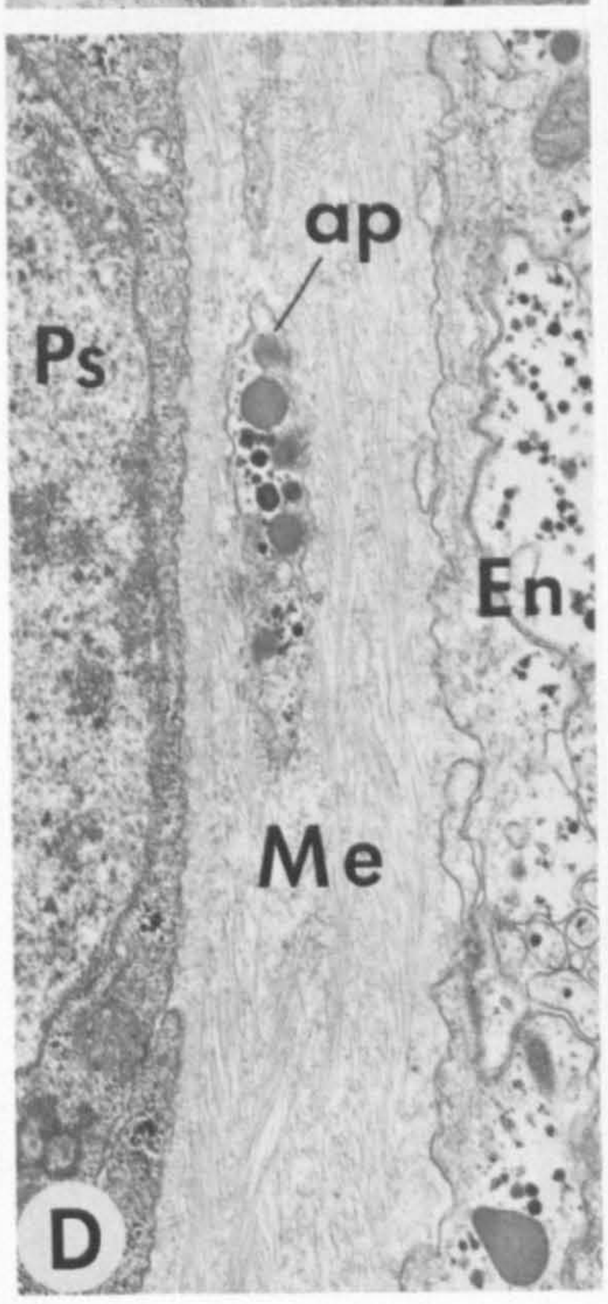
The prospermatogonia generally form groups within the endoderm before they enter the mesoglea. How these groups arise is not clear. It seems likely that they arise by mitosis of individual prospermatogonia, but confluence of individual cells to form groups may well also occur. Mitosis was not observed among endodermal prospermatogonia, but then it was only rarely observed in growing testicular cysts in the mesoglea, where presumably mitosis must occur frequently. It has been suggested that mitosis may be of short duration in sea anemones [Doumenc, 1977] and so is only rarely seen in random sections. In contrast, Schincariol and Habowsky [1972] were able to describe mitosis in the primary spermatogonia of Hydra fusca in some detail. In some endodermal groups in A fragacea, the prospermatogonia are closely

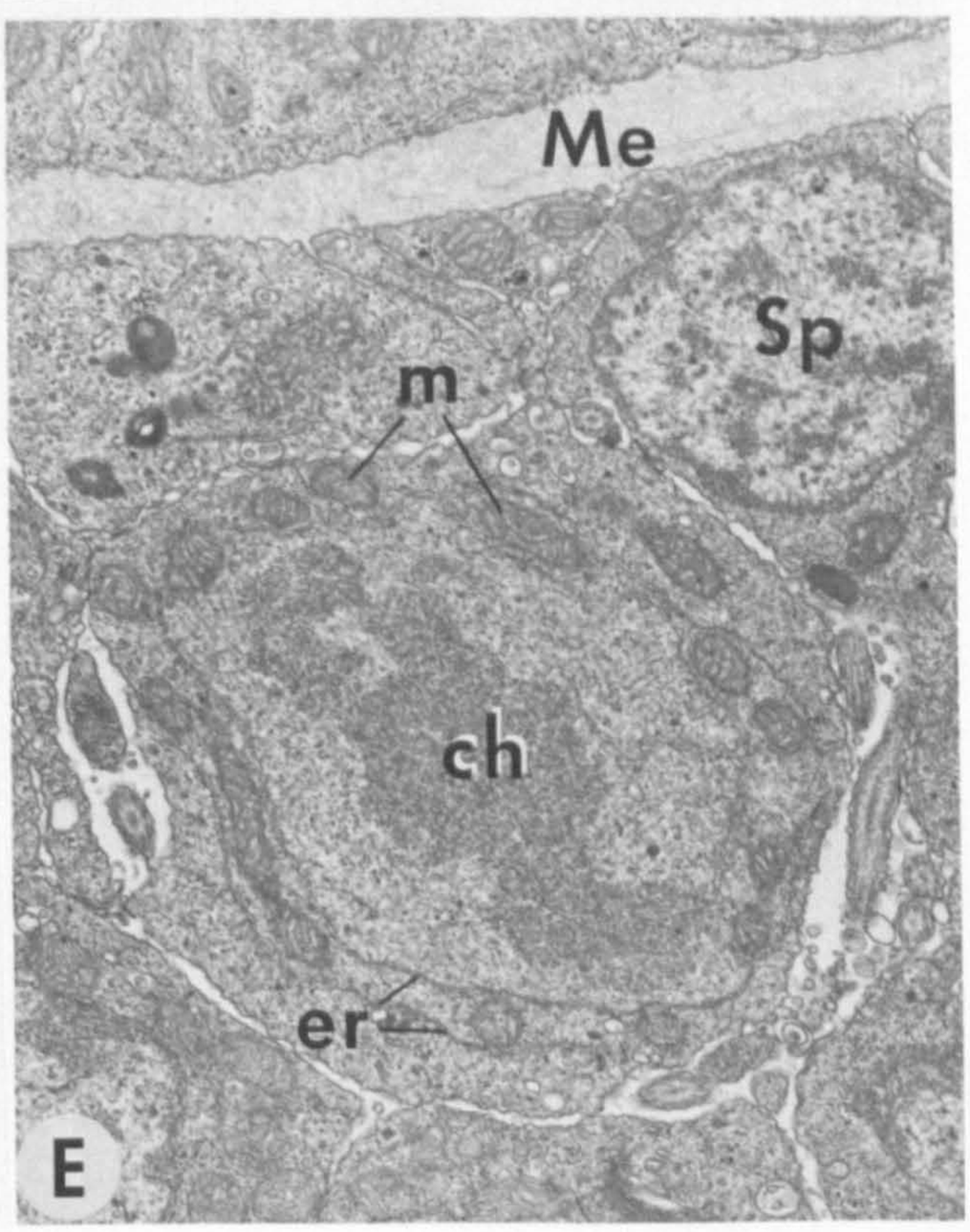
Fig. 11. A. A "light cell" (Lc) within a group of prospermatogonia (Ps) that have nearly completed migration from the endoderm (En) into the mesoglea. B. A "light cell" showing the very low electron density of the nucleus (N). The cytoplasm contains normal-looking mitochondria (m) but few other organelles, and the cell membrane is disrupted at several points (arrowed). ×10,000. C. Amoebocytes (Ac) lying around a group of prospermatogonia (Ps) during entry. They lie between the fibrous mesoglea (Me) and the basal lamina (bl) around the germ cells. ×9,000. D. The layer of mesoglea (Me) around a group of prospermatogonia (Ps) during entry, showing the closely packed collagen fibrils. A slender amoebocyte process (ap) is also visible. En, endoderm. ×15,000. E. Part of a group of spermatogonia (Sp) in the mesoglea (Me) after completing entry. The cell in the centre is undergoing mitosis. The chromatin (ch) is arranged as an irregular band across the cell, perhaps indicative of metaphase, and the mitochondria (m) and endoplasmic reticulum (er) are arranged around the nuclear area. ×13,000.

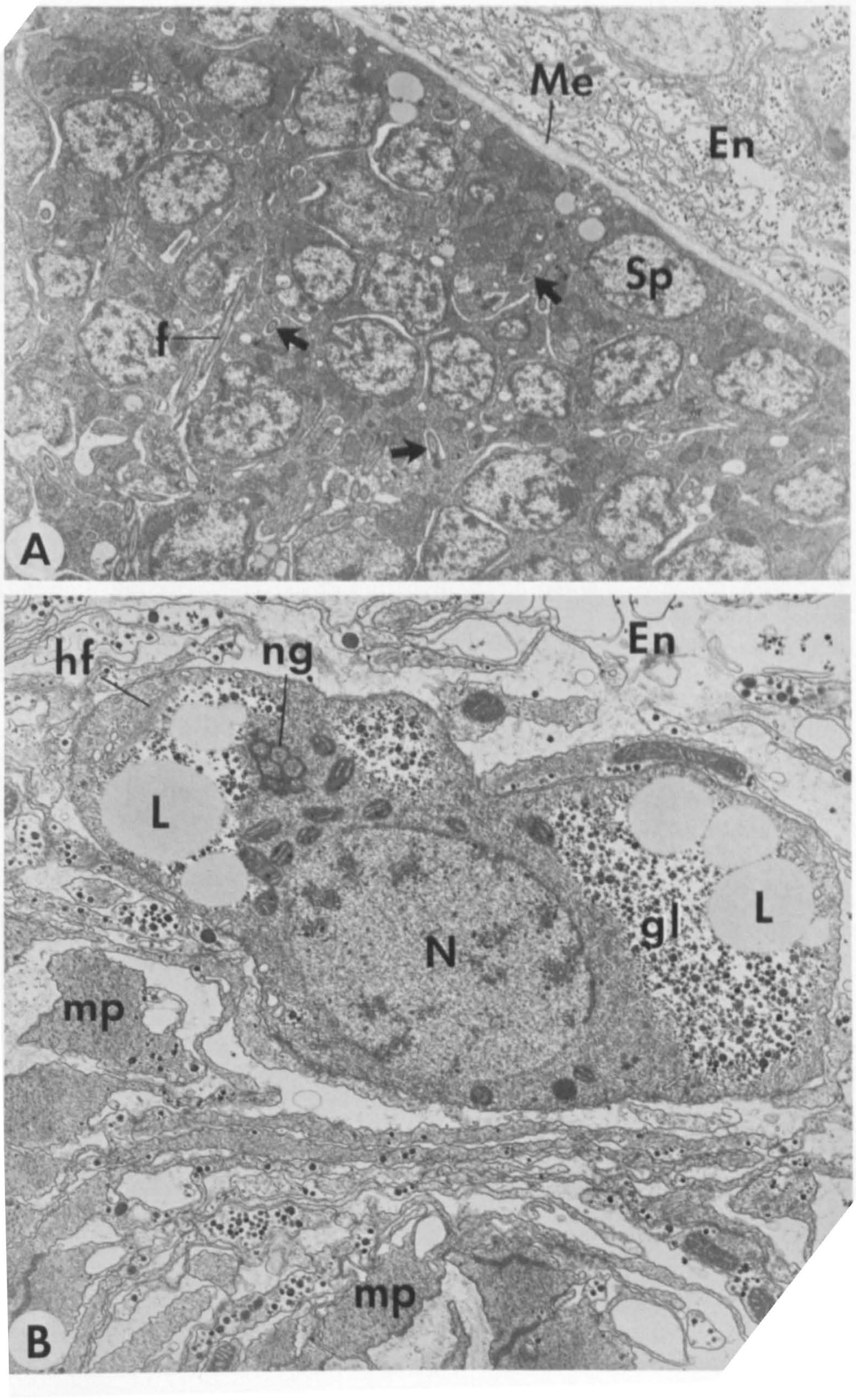

En
Ps.
Lc
A


N
m
B


Me
Ac
bl
Ps
C


ap
Ps
En
Me
D


Me
Sp
m
ch
er
E


Me
En
Sp
f
A
hf
ng
En
L
L
gl
N
mp
mp
B

packed and separated by only narrow intercellular gaps. In other groups, however, slender processes from the surrounding epithelial cells extend between the germ cells. This might suggest that the cells in these groups had migrated towards each other rather than forming as the result of mitosis. Alternatively, the epithelial processes might push their way between the daughter cells after mitosis. Many of the processes contain bundles of microfilaments and microtubules, and so could be capable of such movements.

The spermatogonia of many animal species including mammals tend to be connected by intercellular bridges [Dym and Fawcett, 1971]. Such bridges have also been reported between Hydra spermatogonia [Stagni and Lucchi, 1970]. These bridges are the result of incomplete cytokinesis after cell division, and may serve to synchronize the development of conjoined cells. In Actinia, intercellular bridges occur between spermatids [Larkman, 1980], but have not been observed between spermatogonia. Several prospermatogonia within a group sometimes appear to radiate from a common focus, and pairs of cells may give the impression of having only recently separated, but cytoplasmic continuity between prospermatogonia has not been observed. The significance of the vesicles and undulating membranes found between some cells in groups is not understood. Thus, from the present study, it is not possible to ascertain whether the groups of cells that migrate into the mesoglea are clones arising from the division of a single early germ cell, or are aggregations of cells from different clones.

After the endodermal phase, the groups of prospermatogonia enter the mesoglea, although a small proportion of cells may enter singly. The migration of groups of male germ cells into the mesoglea has been reported for the anemone Anthopleura elegantissima by Jennison [1979]. The method of movement of these "slugs" of cells is not clear, but their behaviour is similar in many ways to that of oocytes that enter the mesoglea of the female gonad individually. In both cases, the cells display no obvious specializations either for cell locomotion or for penetrating the mesoglea. Slugs of prospermatogonia often become constricted into hourglass shapes as they enter, a situation often encountered during oocyte entry [Dunn, 1975; Larkman, 1983]. Mesogleal outgrowth around the entering cells appears to play a significant part in the entry process in both male and female, although the mechanism of this observed outgrowth is unknown. Both male "slugs" and oocytes become covered by the basal lamina as they enter the mesoglea. Presumbly, extra basal lamina must be added as the cells progressively enter and then enlarge within the mesoglea, but in both cases the source of this material is uncertain. Schmidt and Schäfer [1980] termed the extracellular coat around anthozoan mesogleal oocytes a vitelline membrane. However, since spawned A fragacea eggs have no extracellular coat [unpublished observation], and since similar material occurs around both mesogleal oocytes and

Fig. 12. A. Part of a developing testicular cyst containing numerous spermaotognia (Sp), which are separated from the endoderm (En) by only a thin layer of sparsely fibrous mesoglea (Me). Many flagella (f) can be seen within the cyst, often lying in cytoplasmic channels (arrowed). ×4,000. B. A large germ cell remaining near the muscle processes (mp) of the endoderm (En) long after most prospermatogonia have entered the mesoglea. The nucleus (N) is of more uniform density than usual, and the cytoplasm contains extensive reserves of lipid (L) and glycogen (gl), as well as nuage (ng) and hooped fibrillar structures (hf). ×13,000.

groups of male germ cells, the term vitelline membrane, for A fragacea at least, is perhaps inappropriate.

Different authors have used various terms for the mesogleal groups of male germ cells. Dunn [1975] described spermatogenesis occurring in "follicles," Jennison [1979] in "vesicles," while Dewel and Clark [1972] and West [1980] used the term "testicular cyst," as has been used in the present paper. Chia and Rostron [1970], working with Actinia equina, described sperm developing in elongate tubules, reminiscent of the seminiferous tubules of vertebrates. In A fragacea, spermatogenesis takes place within roughly spherical cysts rather than in tubules. My own observations on A equina collected from the same site as the A fragacea used in this study, indicate that in this species also sperm development takes place in spherical cysts, which closely resemble those of A fragacea [Larkman, 1980].

Schmidt and Höltken [1980] indicate that the cyst formed after the male germ cells have entered the mesoglea is closed and completely covered by mesoglea. This differs from the situation in the female where the mesoglea does not overgrow the last part of the oocyte to enter [Schmidt and Schäfer, 1980]. In A fragacea, however, the situation in the male appears to correspond very closely to that in the female. In female A fragacea, the last part of the oocyte to enter the mesoglea retains close contact with the endoderm. This prevents mesogleal overgrowth in this region, and appears to be important in the establishment of a structure known as the trophonema [Larkman, 1983]. The trophonema provides a region of intimate contact between oocyte and endoderm that persists throughout oogenesis. It is active in the uptake and incorporation of radiolabelled precursors from solution, and so is likely to be involved in the transfer of nutrients to the growing oocyte [Larkman and Carter, 1982]. These authors also indicated that a structure corresponding to the trophonema existed in the male gonad, and showed a similarly high level of activity in radioactive tracer uptake. The present study confirms the suggestion that trophonemata occur in both male and female gonads. The male trophonema appears to arise in very much the same way as in the female. The last part of the "slug" of prospermatogonia to enter the mesoglea does not detach from the endodermal cell bases as it enters but rather drags them into the mesoglea behind it. These endodermal cell bases prevent mesogleal overgrowth from sealing off the developing cyst, and the endodermal cells in contact with the germ cells begin to differentiate into specialized trophonemal cells. These cells may be partially separated from the rest of the endoderm by the mesogleal outgrowth round the "slug" during entry, and this may be important in promoting their specialization. Soon after entry, endodermal nuclei are often found close to the region of contact with the germ cells. This suggests that the endoderm is no longer a simple columnar epithelium in this region, since most gonadal epithelial cells have apically situated nuclei, and might indicate that specialization of this region has begun.

In the male, as in the female, the trophonema provides a localized area of close contact between the germ cells and the endoderm that persists after entry into the mesoglea is otherwise complete. In the female, this region is stabilized by the formation of intercellular junctions between oocyte and endoderm, and the area of contact is increased by cytospines or large microvilli that project from the surface of the oocyte and interdigitate with the endodermal cell bases [Larkman, 1983]. No such elaborations appear to exist in the male, however. Close membrane contact between the endoderm and the germ cells does occur, but intercellular junctions or other specializations have not been observed.

In some members of the class Hydrozoa, spermatogenesis takes place in the ectodermal cell layer, among the bases of the epithelial cells. Long epithelial cell processes may traverse the developing spermary, and the germ cells often develop in direct contact with ectodermal cells in this way [Campbell, 1974; Roosen-Runge, 1977]. In A fragacea, most male germ cells appear to develop without direct contact with cells of any other type. Contact with the overlying epithelial cells is restricted to the localized area of the trophonema, and only a small proportion of the germ cells are involved. Occasionally, granular amoebocytes are found inside testicular cysts, where they may play a scavenging role, but they do not enter into a close association with any of the germ cells.

Schmidt and Höltken [1980] suggest that all the cells within a testicular cyst (presumably only up to the spermatid stage) are in contact with the surrounding mesoglea, often by means of long cytoplasmic processes. Thus the cyst could be said to have a complex pseudostratified epithelial structure. In A fragacea, while the groups of germ cells in the mesoglea are small, each cell probably is in contact with surrounding basal lamina. In larger cysts, many of the germ cells may be irregular in shape and may possess long processes. Fine cytoplasmic profiles are often seen against the basal lamina, but it is not proved possible to trace connections between cells near the centre of the cysts and the basal lamina. Thus at present it is not possible to confirm or deny Schmidt and Höltken's suggestion, but it remains an intriguing possibility.

The finding of granular amoebocytes flattened around developing testicular cysts is unexplained, but has a close parallel in the female gonad. Amoebocytes are found around growing oocytes in an exactly corresponding position between the basal lamina and the fibrous mesoglea [unpublished observation]. In both cases, the amoebocytes do not entirely surround the germ cells and are so unevenly distributed that it is difficult to envisage an essential role for them during gametogenesis. Their position in both sexes perhaps provides another example of the equivalence which seems to exist in a number of respects between a cyst or group of germ cells in the male gonad and a single developing oocyte in the female.

The occasional occurrence of cells of much lower electron density among groups of normal-looking prospermatogonia has not been remarked by previous authors. Perhaps the point should be made that the normal spermatogonia shown by Schmidt and Höltken [1980] are generally of much lower density than those found in the present study. In A fragacea, many of these "light cells" contain axonemes and structures resembling nuage material, so it seems likely that they are of germ cell origin. They usually appear degenerate, and so may represent prospermatogonia that for some reason fail to develop normally and break down. Possibly they derive from the "shrunken cells" that are occasionally seen in prospermatogonial groups. The loss or degeneration of a proportion of the germ cells at some stage during spermatogenesis is common in a wide range of animal species [Roosen-Runge, 1977].

The finding of occasional germ cells apparently left behind in the endoderm long after the majority have entered the mesoglea is unexplained but raises some interesting possibilities. All that can be said with any certainty is that there appears to exist a small minority of germ cells whose behaviour and appearance differ from the rest. One possibility is that these cells are merely defective and fail to follow the normal developmental programme and eventually degenerate. None of this type of cell seen so far has shown any signs of degeneracy, however. Another possibility is

that a small number of germ cells are left behind in the endoderm each year, and remain there to provide a reserve that could give rise to the following season's crop of prospermatogonia. Most of the germ cells left behind in this way are larger than the normal prospermatogonia, and they may contain considerably more reserve materials in the form of glycogen and lipid droplets. In this respect, and in their appearance generally, they very closely resemble oocytes of comparable size [see Larkman, 1983]. This suggests a third possibility, that these cells could initiate a female reproductive cycle on some subsequent occasion. However, studies continued from those of Carter and Funnell [1980] have provided evidence that A fragacea individuals maintained in the laboratory have not changed sex during a 3-year period.

Until more is known about the cellular origins of the germ cells in A fragacea and other sea anemones, including the possible role of interstitial cells, many of the anomalies encountered during the present work seem likely to remain unexplained.

ACKNOWLEDGMENTS

The author is grateful to Dr. M.A. Carter for helpful discussion and critical reading of an earlier draft of the paper and to Mrs. L. Healey for typing the manuscript.

REFERENCES

Bode HR, David CN (1978): Regulation of a multipotent stem cell, the interstitial cell of hydra. Prog Biophys Mol Biol 33:189–206

Campbell RD (1974): Cnidaria. In Giese AC, Pearse JS (eds) "Reproduction of Marine Invertebrates, Vol 1." New York: Academic Press, pp 133–199.

Carter MA, Funnell ME (1980): Reproduction and brooding in Actinia. In Tardent P, Tardent R (eds): "Developmental and Cellular Biology of Coelenterates." Amsterdam: Elsevier/North Holland Biomedical Press, pp 17–22

Carter MA, Thorpe JP (1981): Reproductive, genetic and ecological evidence that Actinia equina var. mesembryanthemum and var. fragacea are not conspecific. J Mar Biol Assoc UK 61:79–93

Chapman DM (1974): Cnidarian histology. In Muscatine L, Lenhoff HM (eds): "Coelenterate Biology. Reviews and New Perspectives." New York: Academic Press, pp 2–92

Chia FS, Crawford BJ (1973): Some observations on gametogenesis, larval development and substratum selection of the sea pen Ptilosarcus guerneyi. Mar Biol 23:73–82

Chia FS, Rostron MA (1979): Some aspects of the reproductive biology of Actinia equina (Cnidaria: Anthozoa). J Mar Biol Assoc UK 50:253–264

Chia FS, Spaulding JG (1972): Development and juvenile growth of the sea anemone Tealia crassicornis. Biol Bull 142:206–218

Clark WH, Dewel WC (1974): The structure of the gonads, gametogenesis and sperm-egg interactions in the Anthozoa. Am Zool 14:495–510

Dewel WC, Clark WH (1972): An ultrastructural investigation of spermiogenesis and the mature sperm in the anthozoan Bunodosoma cavernata (Cnidaria). J Ultrastruct Res 40:417–431

Doumenc DA (1977): Etude dynamique de la morphogénèse au course des phases Actinella et Edwardsia de l'actinie Cereus pedunculatus Pennant. Arch Zool Exp Gen 118:79–102

Dunn DF (1975): Reproduction of the externally brooding sea anemone Epiactis prolifera Verrill, 1869. Biol Bull 148:199–218

Dym F, Fawcett DW (1971): Further observations on the numbers of spermatogonia, spermatocytes and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 4:195–215

Eddy EM (1975): Germ plasm and the differentiation of the germ cell line. Int Rev Cytol 43:229–280

Hanisch J (1971): Die Blastostyle—und Spermienentwicklung von Eudendrium racemosum Cavolini. Zool Jahrb (Anat) 87:1–62

Hinsch GW (1974): Comparative ultrastructure of cnidarian sperm. Am Zool 14:457–465

Hinsch GW, Clark WH (1973): Comparative fine structure of cnidarian spermatozoa. Biol Reprod 8: 62-73

Jennison BL (1979): Gametogenesis and reproductive cycles in the sea anemone Anthopleura elegantissima (Brandt, 1835): Can J Zool 57:403-411

Kleve MG, Clark WH (1976): The structure and function of centriolar satellites and pericentriolar processes in cnidarian sperm. In Mackie GO (ed): "Coelenterate Ecology and Behaviour." New York: Plenum Press, pp 309-317

Larkman AU (1980): Ultrastructural aspects of gametogenesis in Actinia equina L. In Tardent P, Tardent R (eds): "Developmental and Cellular Biology of Coelenterates." Amsterdam: Elsevier/North Holland Biomedical Press, pp 61-66

Larkman AU (1981): An ultrastructural investigation of the early stages of oocyte differentiation in Actinia fragacea (Cnidaria: Anthozoa). Int J Invertebr Reprod 4:147-167

Larkman AU (1983): An ultrastructural study of oocyte growth within the endoderm and entry into the mesoglea in Actinia fragacea (Cnidaria: Anthozoa). J Morphol 178:155-177

Larkman AU, Carter MA (1980): The spermatozoon of Actinia equina L. var. mesembryanthemum. J Mar Biol Assoc UK 60:193-204

Larkman AU, Carter MA (1982): Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone Actinia fragacea has a nutritive function. Int J Invertebr Reprod 4:375-379

Lyke EB, Robson EA (1975): Spermatogenesis in Anthozoa: Differentiation of the spermatid. Cell Tissue Res 157:185-205

Nieuwkoop PD, Satasurya LA (1981): "Primordial Germ Cells in the Invertebrates." Cambridge: Cambridge University Press, Ch 4, pp 21-36

Rinkevich B, Loya Y (1979): The reproduction of the Red Sea coral Stylophora pistillata. 1. Gonads and planulae. Mar Ecol Prog Ser 1:133-144

Roosen-Runge EC (1977): "The Process of Spermatogenesis in Animals." Cambridge: Cambridge University Press.

Roosen-Runge EC, Szollosi D (1965): On biology and structure of the testis of Phialidium Leuckhart (Leptomedusae). Z Zellforsch 68:597-610

Schincariol AL, Habowsky JEJ (1972): Germinal differentiation of the stem cell in Hydra fusca: A model system. Can J Zool 50:5-12

Schmidt H, Höltken B (1980): Peculiarities of spermatogenesis and sperm in Anthozoa. In Tardent P, Tardent R (eds): "Developmental and Cellular Biology of Coelenterates." Amsterdam: Elsevier/North Holland Biomedial Press, pp 53-59

Schmidt H, Schäfer WG (1980): The anthozoan egg: Trophic mechanisms and oocyte surfaces. In Tardent P, Tardent R (eds): "Developmental and Cellular Biology of Coelenterates." Amsterdam: Elsevier/North Holland Biomedical Press, pp 41-46

Schmidt H, Zissler D (1979): Die Spermien der Anthozoa und ihre phylogenetische Bedeutung. Zoologica (Stuttg) 44(129):1-98

Stagni A, Lucchi ML (1970): Ultrastructural observations on the spermatogenesis in Hydra attenuata. In Baccetti B (ed): "Comparative Spermatology." New York: Academic Press, pp 357-361

Szmant-Froelich A, Yevich P, Pilson MEQ (1980): Gametogenesis and early development of the temperate coral Astrangia danae (Anthozoa: Scleractinia). Biol Bull 158:257-269

Tiffon Y, Hugon JS (1977): Localisation ultrastructurale de la phosphatase acide et de la phosphatase alcaline dans les cloisons septales steriles de l'anthozoaire Pachycerianthus fimbriatus. Histochemistry 54:289-297

West DL (1980): Spermiogenesis in the anthozoan Aiptasia pallida. Tissue Cell 12:243-253

Protoplasma 122, 203–221 (1984)

PROTOPLASMA

The Fine Structure of Granular Amoebocytes from the Gonads of the Sea Anemone *Actinia fragacea* (*Cnidaria: Anthozoa*)

A. U. LARKMAN*

Department of Biological Sciences, Portsmouth Polytechnic, Portsmouth

Received October 13, 1983
Accepted January 1, 1984

Summary

The structure of granular amoebocytes of the intertidal sea anemone *Actinia fragacea* (*Cnidaria: Anthozoa*) has been investigated using the electron microscope. Cells from the gonads of large, intact individuals were studied in most detail, but other regions of the anemone were also examined. The amoebocytes are cells of variable appearance which are widely distributed both in the mesogloea and in the epithelial cell layers. They contain numbers of characteristic dense granules, which may enclose spherical cores of greater or lesser electron density. They also contain rough endoplasmic reticulum, Golgi apparatus and a range of inclusions, some of which may have lysosomal origins. They may contain extensive deposits of glycogen, and usually smaller quantities of lipid droplets. They may take on a variety of forms, depending partly on their location within the various types of mesogloea and epithelia. The amoebocytes appear to be motile and phagocytic, and may also be involved in the storage and transport of glycogen. They are involved with gametogenesis, both during the development of the oocytes and spermatogenic cysts and during the resorption of degenerating gametes. Their possible role in the secretion or maintenance of the mesogloea remains uncertain. No evidence of amoebocytes differentiating into other cell types was obtained.

Keywords: *Actinia fragacea*; Granular amoebocytes; Ultrastructure.

1. Introduction

The coelenterate body plan comprises two epithelial cell layers, the ectoderm and endoderm, separated by a layer of mesogloea (HYMAN 1940). In hydrozoan species, the mesogloea tends to be non-cellular. However, in scyphozoans and anthozoans the mesogloea may be thick relative to the cell layers, and it may contain large numbers of cells. While more than one cell type may be found within the mesogloea, the most typical cell is irregular in shape and is usually referred to as an amoebocyte.

The mesogloea and the amoebocytes contained within it were described at the light microscope level for several scyphozoan and anthozoan species by CHAPMAN (1953), who commented on the similarity in appearance of these amoebocytes to the fibroblasts of higher organisms. At the electron microscope level, coelenterate amoebocytes have been mentioned in several investigations since the pioneering work of GRIMSTONE *et al.* (1958), who examined the mesenteries of the sea anemone *Metridium senile*. A number of suggestions have been made as to the function of amoebocytes in *Anthozoa*, and these include the production of mesogloeal collagen fibrils (YOUNG 1974) and spicules (BUISSON and FRANC 1969, FRANC 1970), the transport of nutrients and waste products (YOUNG 1974, VAN PRAET 1978), and participation in an inflammatory response (PATTERSON and LANDOLT 1979). Most authors suggest that, at least under some circumstances, amoebocytes can show phagocytic activity (BUISSON and FRANC 1969, FRANC 1970, VAN PRAET 1974, VAN PRAET and DOUMENC 1975, WATSON and MARISCAL 1983). It has also been suggested that amoebocytes can differentiate into other cell types, including nerve cells and mucus producing cells (BUISSON and FRANC 1969, FRANC 1970), and cnidocytes

* Correspondence and Reprints: Department of Biological Sciences, Portsmouth Polytechnic, King Henry 1 Street, Portsmouth, PO1 2DY, Hampshire, United Kingdom.

(CHAPMAN 1974). In a review of coelenterate histology, CHAPMAN (1974) compares and contrasts the structure and role of the amoebocytes of scyphozoans and anthozoans with the interstitial cells of hydrozoans. The interstitial cell system of *Hydra* and some other hydrozoans has been the subject of considerable research interest in recent years (see BODE and DAVID 1978, TARDENT and TARDENT 1980). Amoebocytes, however, have received much less attention, and, as CHAPMAN (1974) pointed out, no detailed ultrastructural description of an anthozoan amoebocyte has yet been published.

This paper describes the ultrastructural appearance of granular amoebocytes from the gonads of the intertidal sea anemone *Actinia fragacea*. The gonads were selected for detailed study principally because large quantities of this material were available from a study of gametogenesis in this species (LARKMAN 1980, 1981, 1983). Mesenteries and filaments were also examined in some detail, while body wall and tentacle tissue were scanned more briefly. A preliminary investigation and a previously published report (MINASIAN 1980) had indicated that granular amoebocytes were abundant in the mesenteries generally.

Actinia fragacea was until recently thought to be a variety of the species *Actinia equina*. CARTER and THORPE (1981), however, have presented evidence that the two are separate species, and their suggested classification has been followed in this paper.

2. Materials and Methods

Large individuals of *Actinia fragacea* were collected from the intertidal zone of the rocky shore at Wembury, near Plymouth, Devon, at regular intervals throughout 1979 and 1980. The anemones were dissected under sea water and small pieces of gonad were removed and fixed by immersion in 3% glutaraldehyde in a 0.1 M phosphate buffer containing 3% sodium chloride. Pieces of filament, mesentery, tentacle and body wall were also taken, although on a less regular basis. The tissue pieces were rinsed in buffer, post-fixed in 1% osmium tetroxide in the same buffer and dehydrated using ethanol before embedding in "Emix" epoxy resin (Emscope Laboratories Ltd.). Sections were cut using glass or diamond knives on an LKB Ultratome III, stained with uranyl acetate and lead citrate and examined using a Philips EM 300 electron microscope. Acid phosphatase enzyme activity was investigated using the Gomori-based method of LEWIS and KNIGHT (1977).

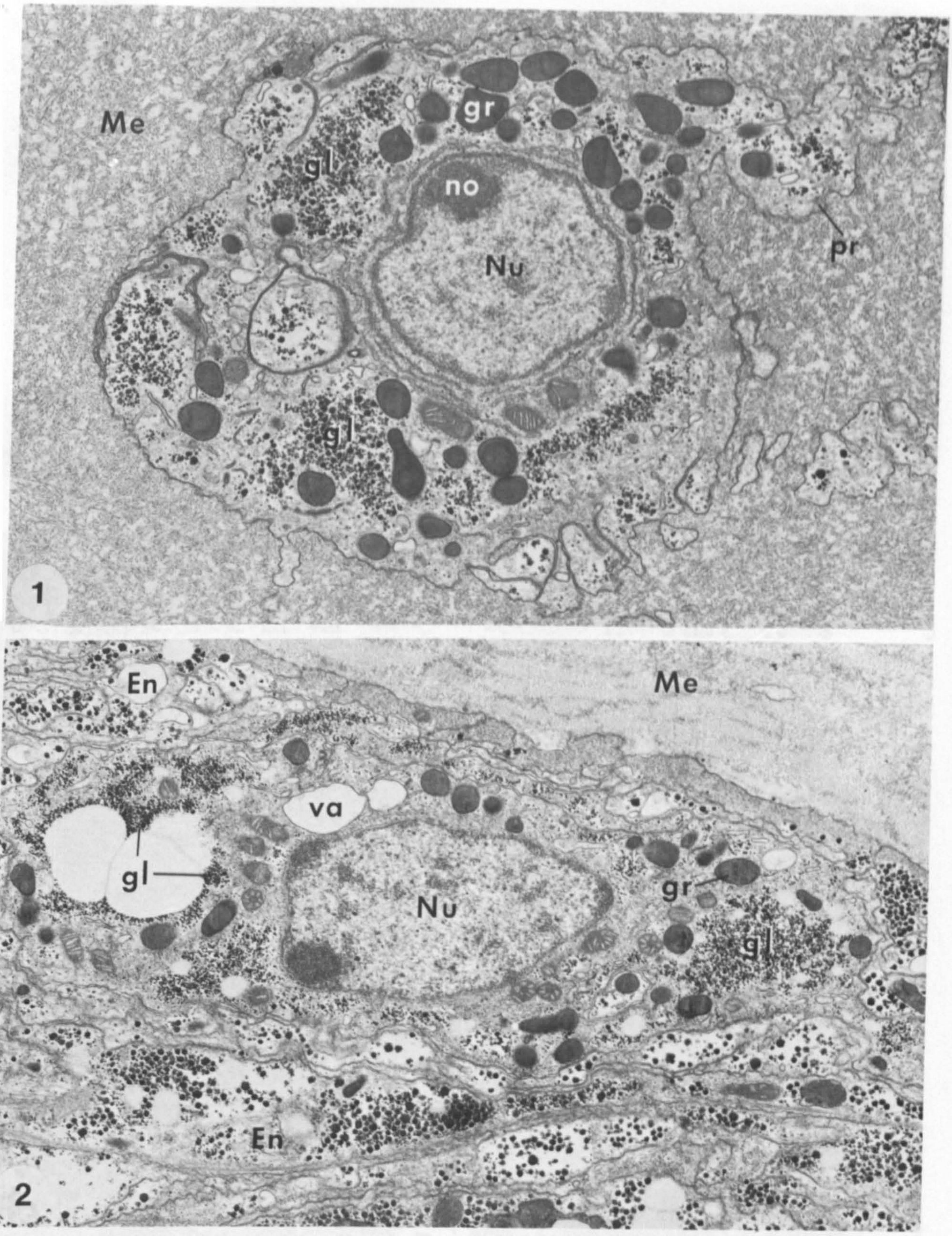
3. Results

The gonads of sea anemones are located on the mesenteries, and lie between the gastric filaments and the mesenteric retractor muscles. Like the rest of the mesentery, they are made up of two layers of endodermal epithelial cells separated by a layer of mesogloea. Non-epithelial cells, including amoebocytes and germ cells, may be found both in the mesogloea and among the bases of the epithelial cells bordering the mesogloea. The epithelial cell bases are made up of many irregular cytoplasmic processes which contain storage materials such as glycogen and lipid droplets, but few other organelles.

Granular amoebocytes were found to be common in the gonads, and present in all regions of the anemone examined. They are cells of often irregular shape and variable appearance, but their nuclear morphology, their rather unspecialized cytoplasm and the presence of numerous characteristic small, dense cytoplasmic granules render them easily distinguishable from other cell types. The general features of granular amoebocytes are shown in Figs. 1 and 2.

Because of their irregular shape, the apparent size of amoebocytes in sections varies greatly. The nucleus is usually spherical or ovoid in shape, some 3–4 µm in diameter, and generally of only moderate electron density (Figs. 1–4). It usually has a smooth or gently scalloped outline, unless the cell is very irregular or contains large inclusions, when the nucleus may be distorted. Rarely, bilobed nuclei are seen (Fig. 3). The typical double membrane nuclear envelope contains a small number of nuclear pores, and its outer aspect is usually studded with ribosomes. Irregular, dense chromatin areas are found scattered throughout the nucleus, and characteristically there is a band of dense material lining the inner aspect of the nuclear envelope. This band is usually continuous, although it may vary in width. One, or rarely two, nucleoli are seen, always situated close to the nuclear envelope, and having a more finely granular appearance than the other chromatin material. Both the nucleoli and the thicker areas of chromatin material are usually situated in outward bulges of the nuclear envelope. The nucleus also contains small granules of more dense material, 30–50 nm in diameter. These granules appear to be randomly distributed within the nucleus, and their number varies from cell to cell.

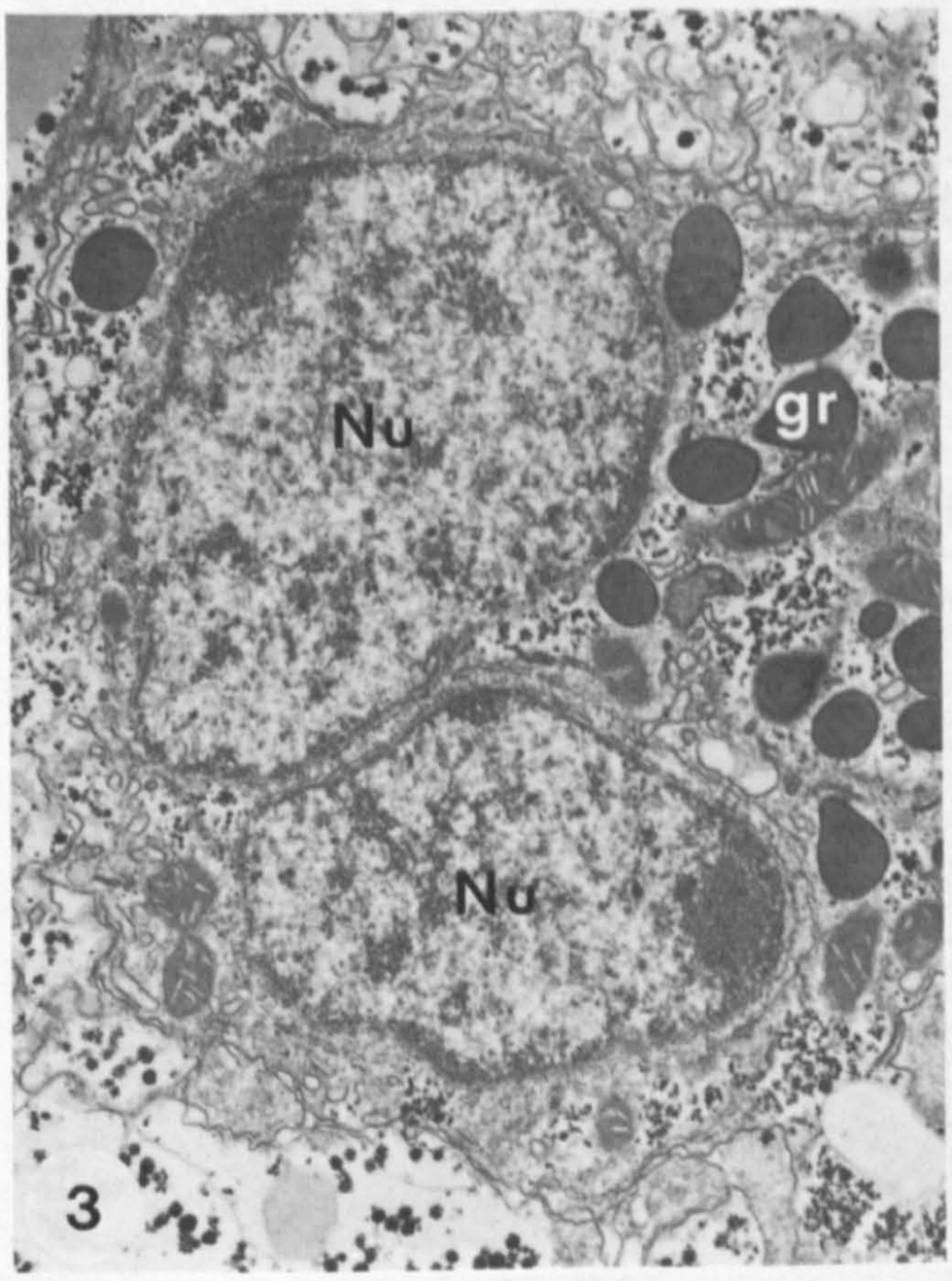
The cytoplasm is usually seen to contain at least a small amount of organized rough endoplasmic reticulum, which may be arranged in two ways. Very often between one and four cisternae of endoplasmic reticulum are found arranged concentrically around the nucleus, completely or partially surrounding it (Fig. 4). Rough endoplasmic reticulum is also found as compact masses in the cytoplasm (Fig. 5), separate from that around the nucleus. These masses may contain up to ten

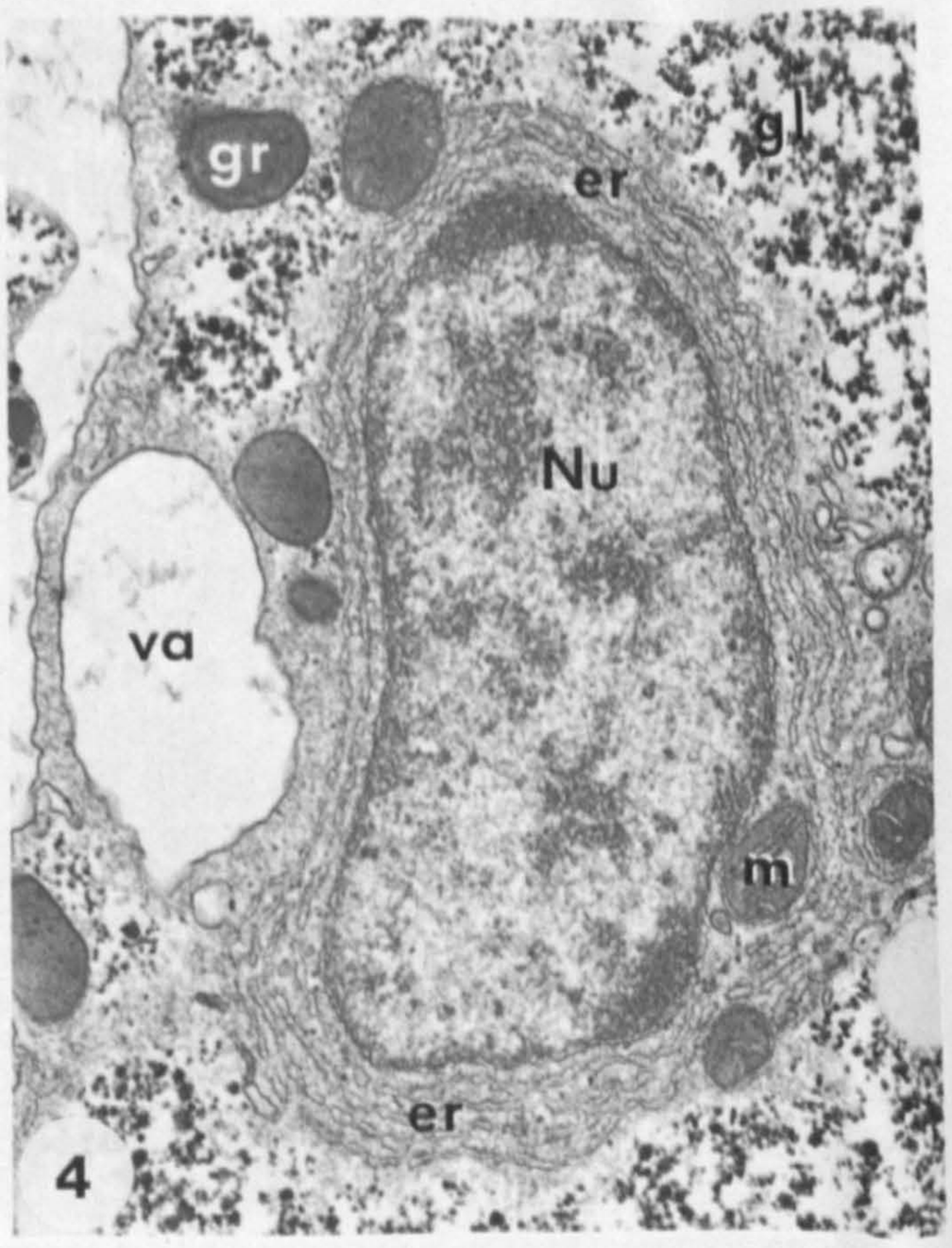


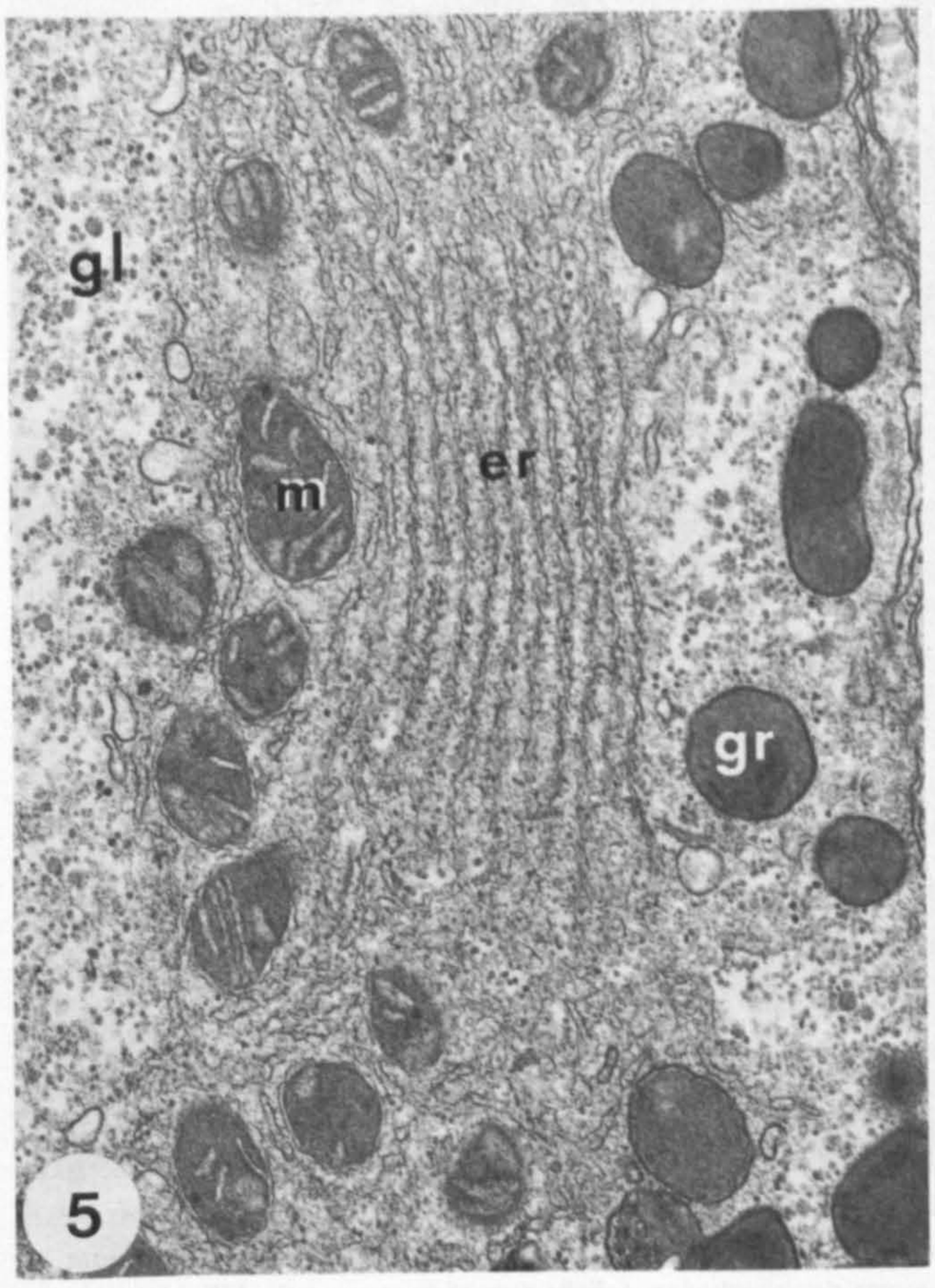
Abbreviations. The following abbreviatons apply to all figures: *ac* angular core of residual body-like inclusion, *bl* basal lamina, *ce* centriole, *cf* collagen fibrils, *cy* cytospines, *db* dense body, *dc* dense core of dense granule, *En* endoderm, *Ep* epithelial cells, *er* endoplasmic reticulum, *ex* expanded form of dense granule, *gl* glycogen, *go* Golgi apparatus, *gr* dense granule, *in* inclusion, *lc* light core of dense granule, *ld* lipid droplet, *m* mitochondrion, *Me* mesogloea, *ne* neck region of glycogen-filled lobe, *no* nucleolus, *Nu* amoebocyte nucleus, *Oc* oocyte, *ph* phagosome, *pl* pleated region, *pr* cytoplasmic process, *rb* residual body-like inclusion, *Sp* spermatogonium, *sr* striated rootlet, *st* striations in dense granule, *va* vacuole, *vs* vesiculated dense granule.

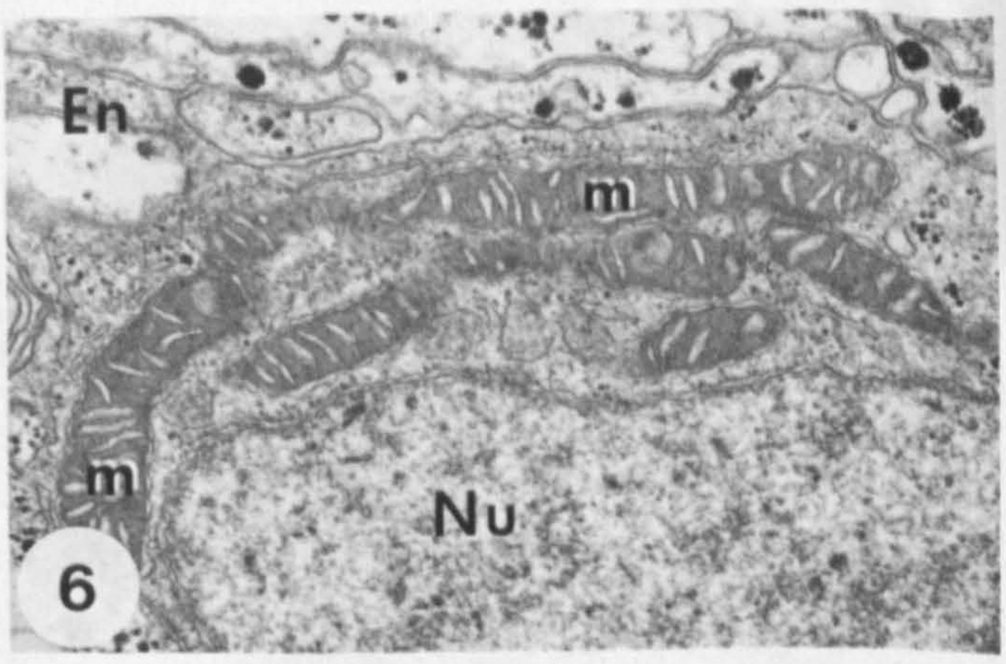
Fig. 1. General view of a granular amoebocyte in the mesogloea of the gonad, showing the typical dense granules and deposits of glycogen. The cell bears a cytoplasmic process (top right). Mag. × 17,000

Fig. 2. Amoebocyte lying among the bases of the endodermal epithelial cells, close to the mesogloea. This cell also contains small, empty vacuoles, and the glycogen deposit to the left surrounds large clear areas. Mag. × 13,500









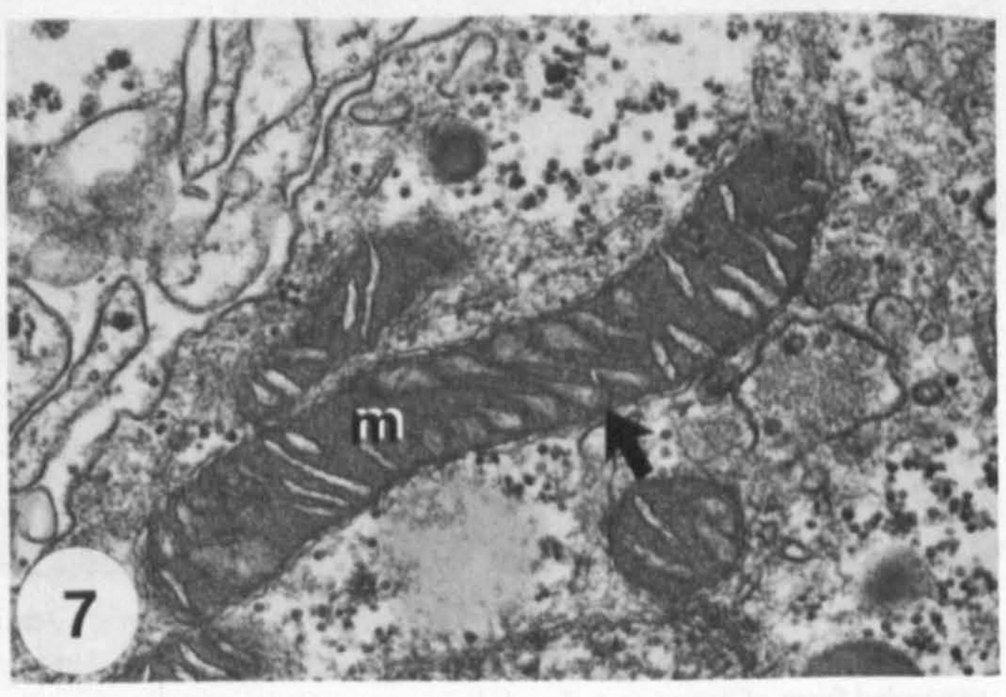


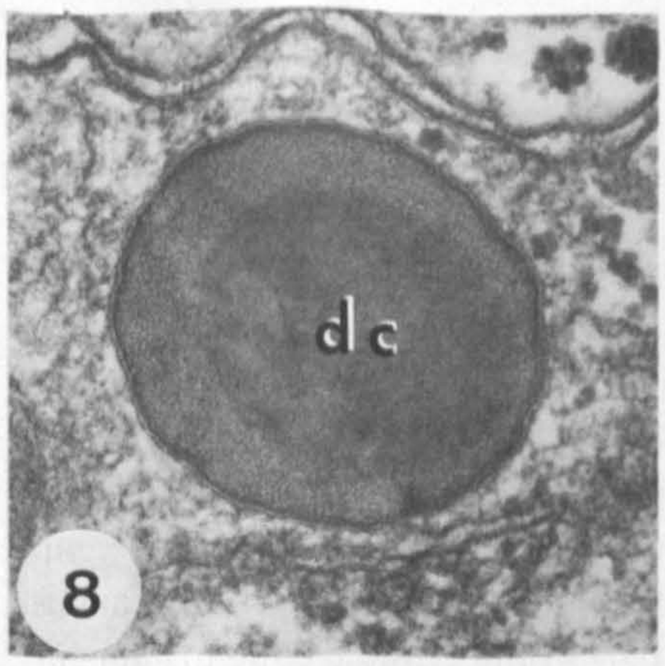
Fig. 3. An amoebocyte with an unusual, apparently bilobed nucleus. Mag. × 16,000

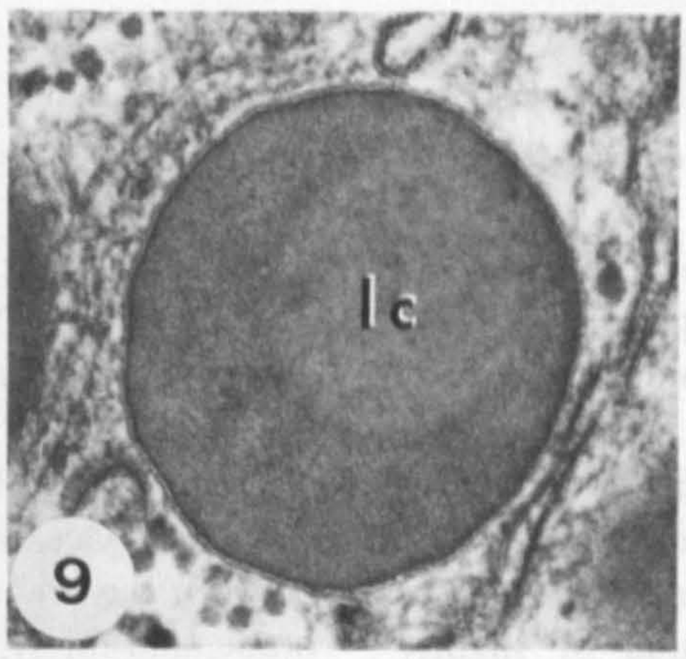
Fig. 4. Amoebocyte with a band of endoplasmic reticulum arranged concentrically around the nucleus. The cell also contains a large vacuole. Mag. × 26,000

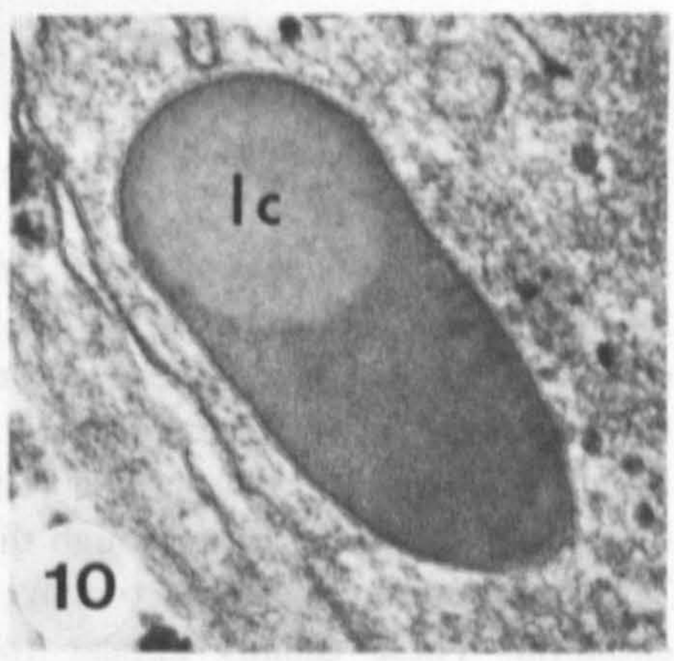
Fig. 5. A stack of rough endoplasmic reticulum in the peripheral cytoplasm, surrounded by glycogen, dense granules and squat, rounded mitochondria. Mag. × 27,000

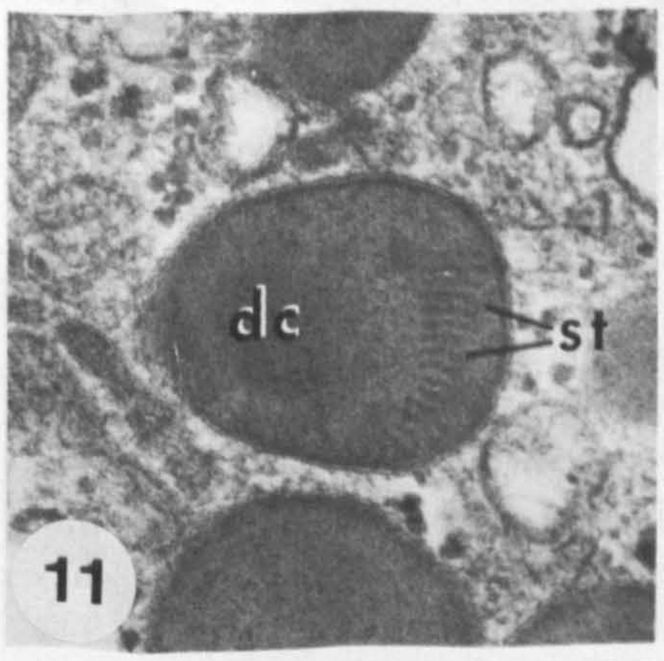
Fig. 6. A group of elongate mitochondria lying next to the nucleus. Mag. × 20,000

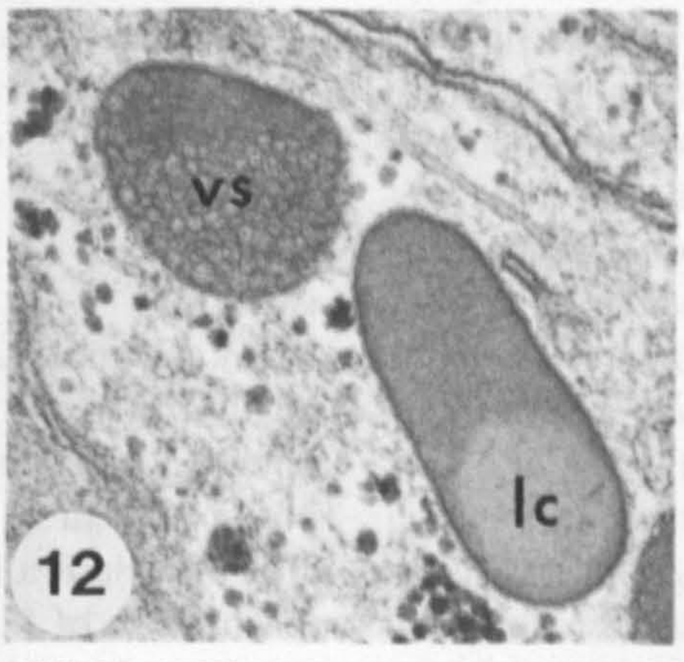
Fig. 7. An elongate mitochondrion with a central region (arrowed) where the cristae are regularly arranged. Mag. × 31,000

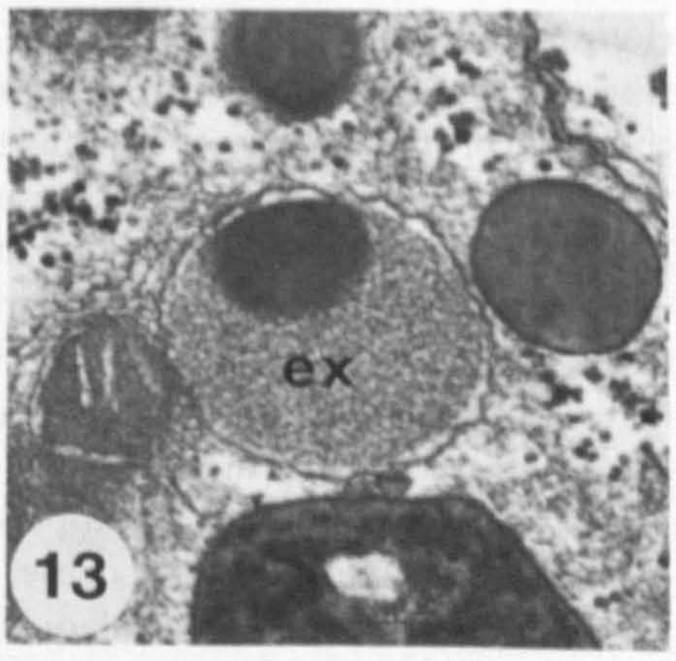


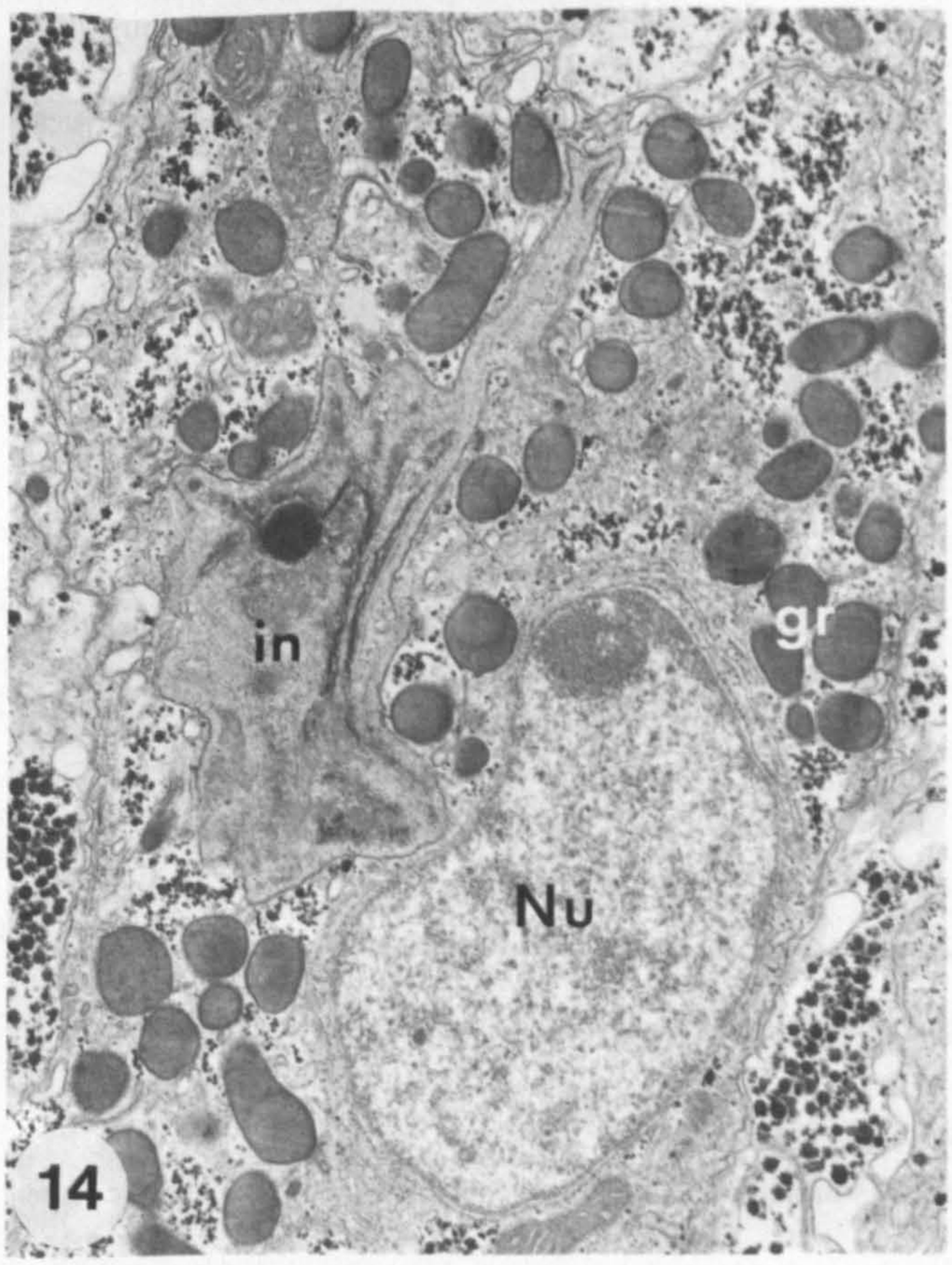












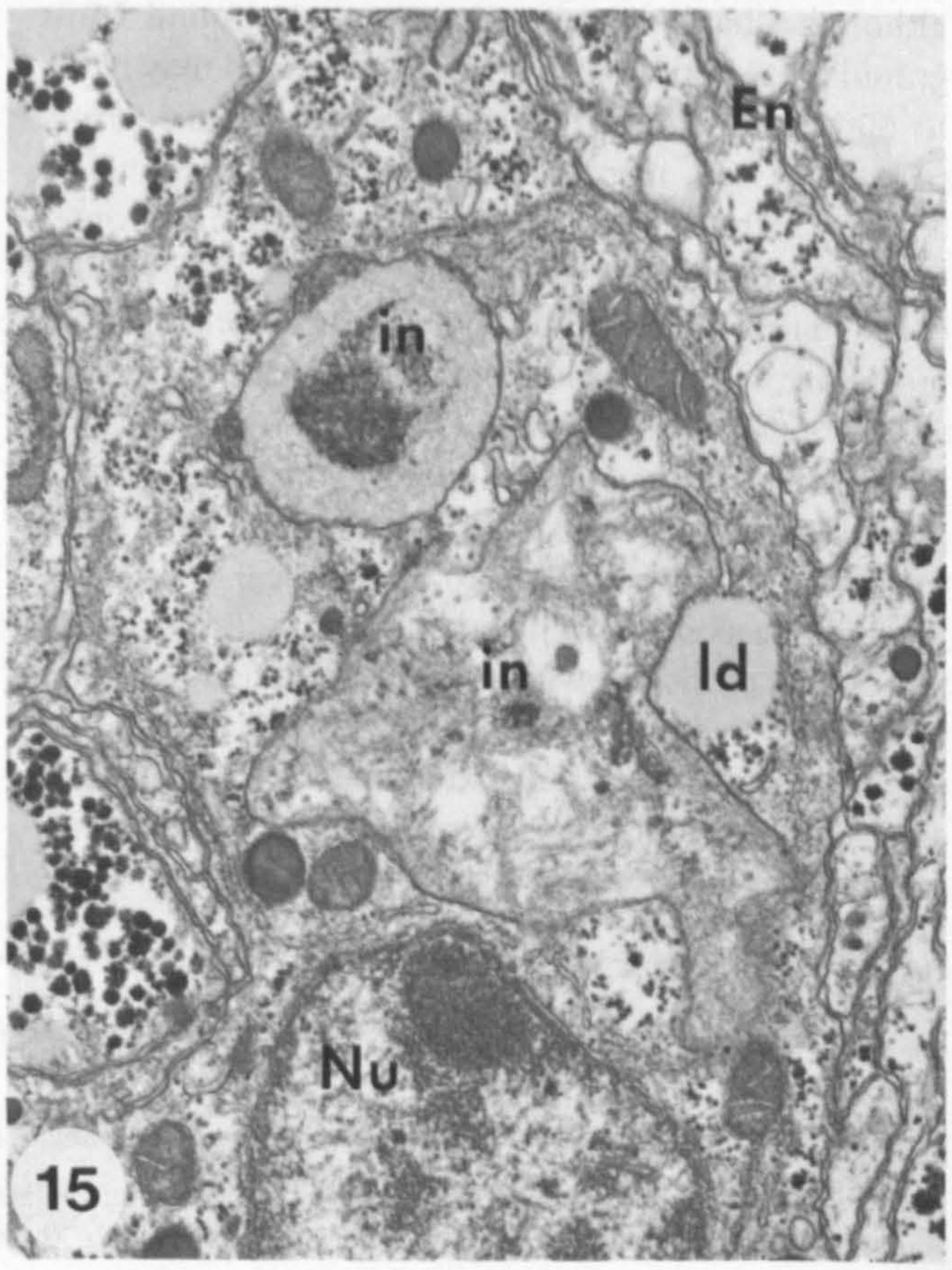


Fig. 8. A typical amoebocyte dense granule, showing the limiting membrane and a core region of greater electron density. Mag. × 125,000

Fig. 9. A dense granule with a core of less dense material. The inner leaf of the trilaminar membrane around the granule appears wider and more dense than the outer. Mag. × 120,000

Fig. 10. Elongate dense granule with a spherical light core located to one end of the granule. Mag. × 100,000

Fig. 11. A granule with a dense core, showing a row of parallel striations beneath the membrane. Mag. × 75,000

Fig. 12. Two granules, one of which appears to be partly composed of small vesicles, while the other shows an eccentric light core. Mag. × 65,000

Fig. 13. A granule, surrounded by a loosely-fitting membrane, in which part of the granule contents appear to be in a more expanded form. Mag. × 45,000

Fig. 14. An amoebocyte containing a large, highly irregular inclusion. Mag. × 17,000

Fig. 15. Two inclusions of rather different appearance. The lower is irregular and similar in composition to that shown in Fig. 14, while the upper is more dense, rounded and compact. Mag. × 23,000

roughly parallel stacked cisternae. In both cases the individual cisternae are narrow and parallel with no obvious dilatations and appear to contain material of low electron density. Short, irregular lengths of both rough and smooth endoplasmic reticulum are also found scattered randomly through the cytoplasm.

The amoebocyte cytoplasm contains numerous mitochondria. These usually appear round or slightly elongate in section, and average 0.3 × 0.7 µm in size (Fig. 5). Occasionally, however, much longer mitochondrial profiles are seen (Fig. 6), up to 3 µm in length, suggesting that at least some of the mitochondria are highly elongate. In all cases, the mitochondrial matrix is dense and homogeneous, and is traversed by numerous narrow cristae. Sometimes, especially in more elongate mitochondria, there may be regions where the cristae show a precise regular arrangement (Fig. 7).

A characteristic feature of the cytoplasm is the presence of numerous small electron dense granules. These usually appear to be spherical or slightly elongate, although other less regular shapes are also found. Most granules are about 300 nm in diameter, and may be up to 500 nm in length. Each granule is bounded by a trilaminar unit membrane, the inner leaf of which appears to be thicker and more dense than the outer (Figs. 8 and 9). The granules contain a finely granular dense material. Some appear to be homogeneous, but most have an inner region or core of different density to the remainder of the granule. This core may appear to be of either greater or lower density than the rest of the granule, and appears more finely granular (Figs. 8 and 9). The core region appears to be spherical, even when the granule containing it is elongate, and it may be located eccentrically within the granule (Fig. 10). In favourable section, some granules show a row of regular parallel striations extending roughly at right angles from the limiting membrane and about 20 nm apart (Fig. 11). A small proportion of granules contain many tightly-packed small vesicles, each some 25 nm in diameter (Fig. 12). These vesicular granules may contain a spherical, finely granular core similar to that seen in granules of more normal appearance. Other granules may contain membranous elements, usually in the form of whorls. Occasionally, there is a narrow clear gap between the granule contents and the limiting membrane, and rarely, part of the granule contents appear to be in a more expanded form (Fig. 13). However, these variants account for only a small fraction of the total. The amoebocyte granules gave only a feebly positive reaction when tested for acid phosphatase enzyme activity, which was not thought to be conclusive.

Many amoebocytes contain one or more inclusions of a highly distinctive appearance. These are membrane-bound, and, although variable in shape and composition, seem to fall into two broad types. The first type of inclusion is highly irregular in shape and may reach a size of 4 µm or more in length (Fig. 14). The contents of these inclusions are very heterogeneous, but the major component appears to be a finely fibrillar material of relatively low electron density. Sometimes this material is seen in an expanded form, interspersed with clear areas. These inclusions may also contain membranous elements, dense bodies and dense granular material. The second type of inclusion is more rounded in shape, and generally smaller, averaging approximately 1 µm in diameter (Fig. 15). They contain irregular areas of electron dense granular material separated by less dense fibrillar material similar to that found in the first type of inclusion. Both types are often found together in the same cell, and may represent different forms of the same structure. Similar inclusions were found in the gonad epithelial cells and the phagocytic absorptive cells of the gastric filament.

Each amoebocyte is usually seen to contain a single prominent Golgi apparatus, consisting of a stack of

---

Fig. 16. Amoebocyte Golgi apparatus, associated with a single cisterna of endoplasmic reticulum, and a small granule with a mottled appearance (arrowed). Mag. × 45,000

Fig. 17. Golgi apparatus associated with two larger mottled granules (arrowed). Mag. × 26,000

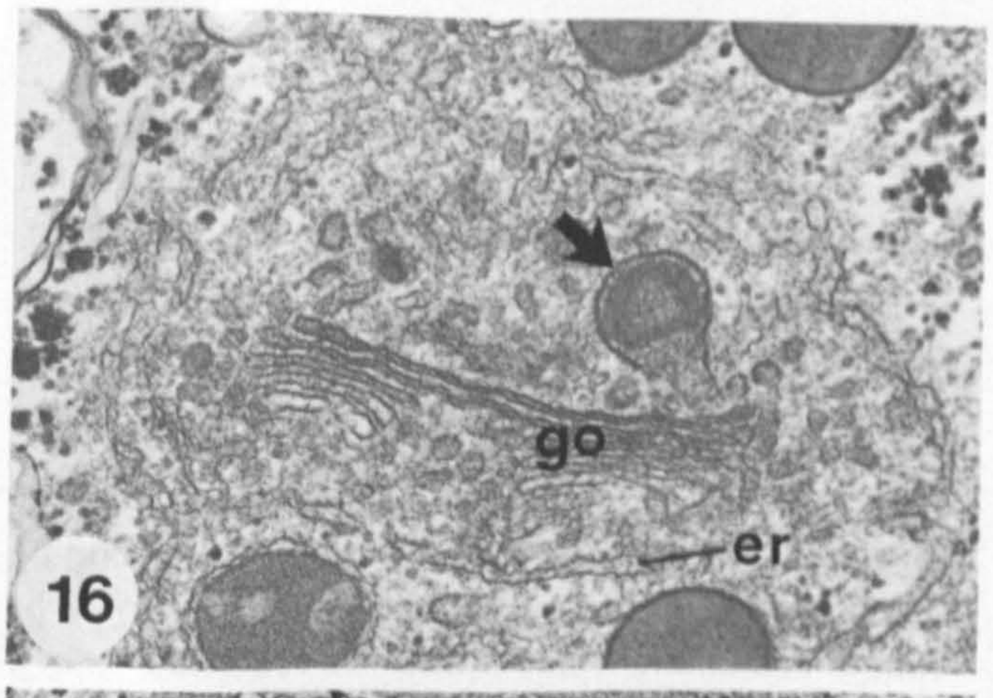
Fig. 18. Golgi apparatus associated with several small dense granules (arrowed), which resemble smaller versions of the typical amoebocyte dense granules. A centriole is also situated close to the Golgi. Mag. × 27,000

Fig. 19. A basal-body-rootlet complex within an amoebocyte, consisting of two centrioles at right angles to each other, attached to a striated rootlet. Mag. × 30,000

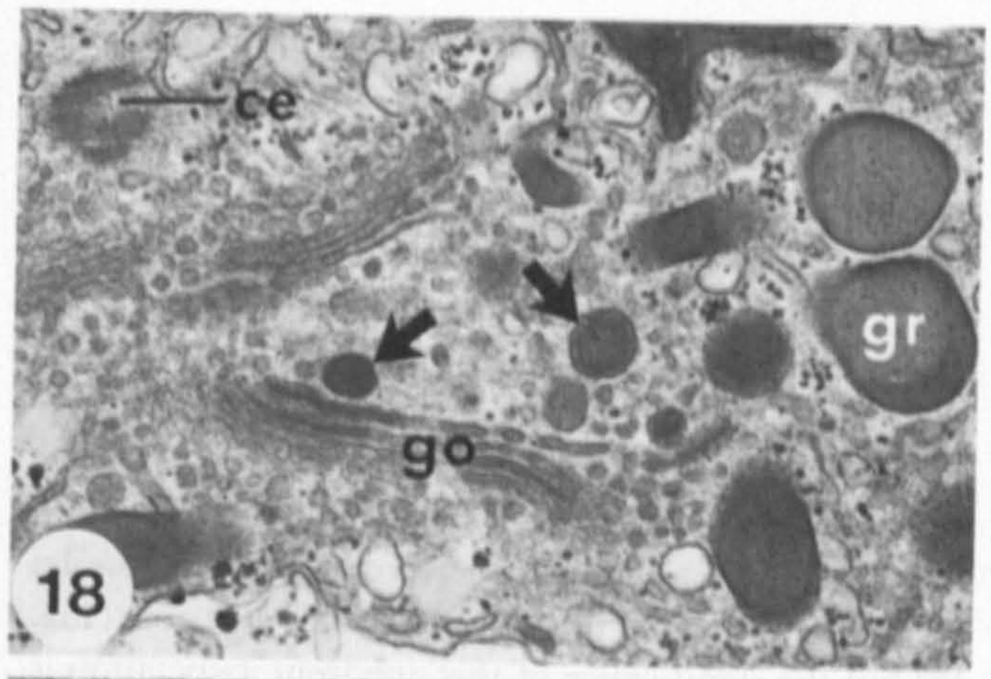
Fig. 20. An amoebocyte containing several residual body-like inclusions. Mag. × 11,000

Fig. 21. Two residual body-like inclusions, showing the characteristic angular electron lucent cores distributed in a highly electron dense matrix. Mag. × 24,000

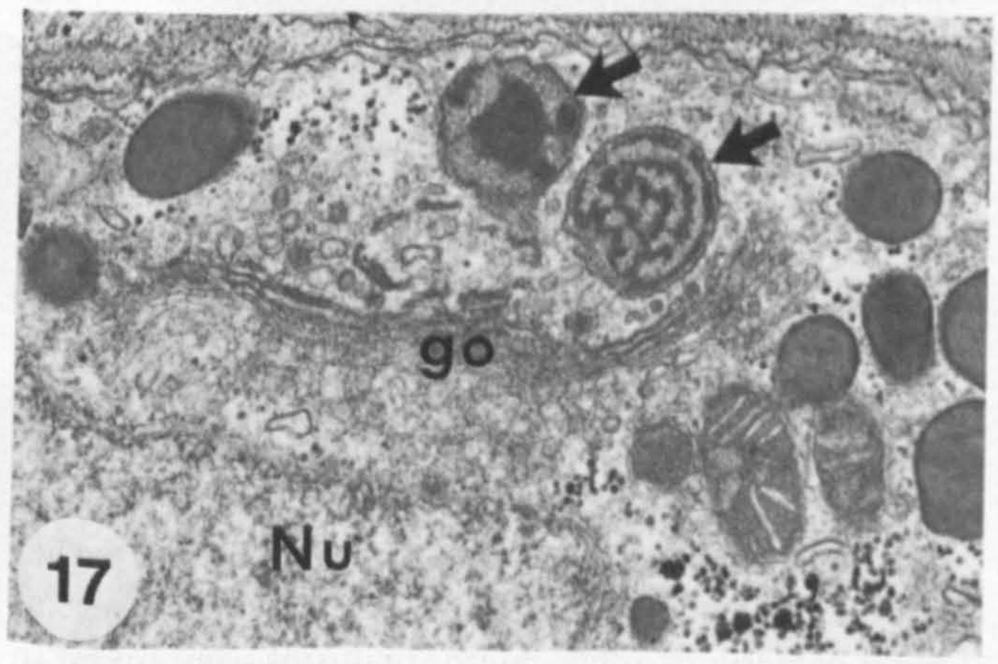
Fig. 22. An unusual residual body-like inclusion, containing a single needle-like angular core. Mag. × 36,000



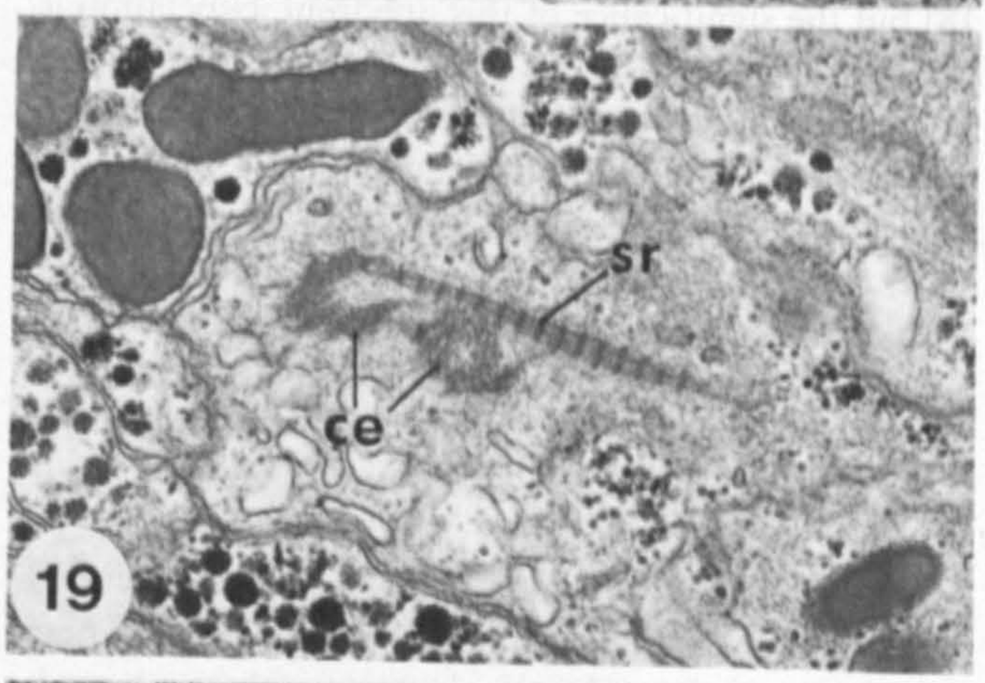
16



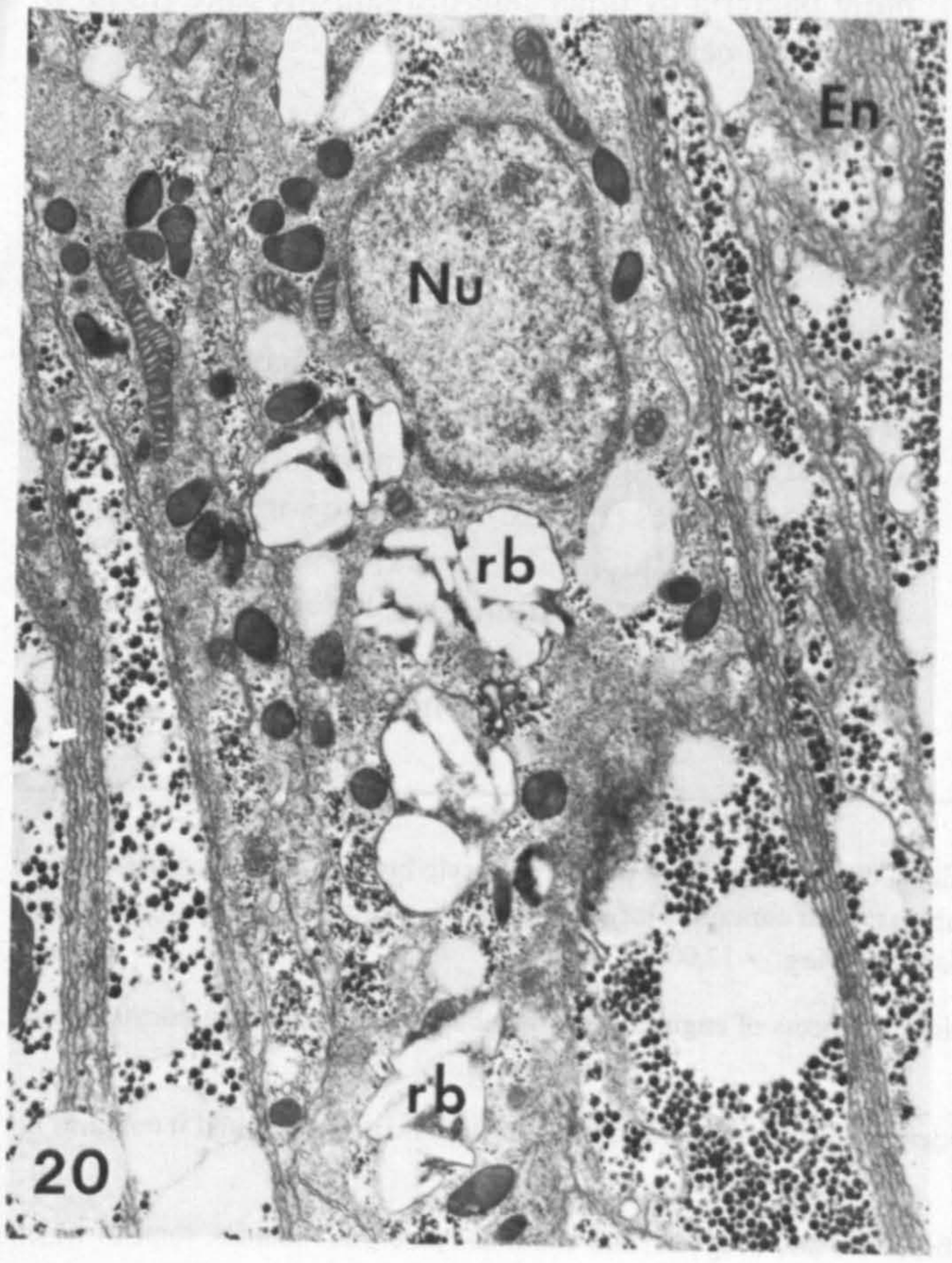
18



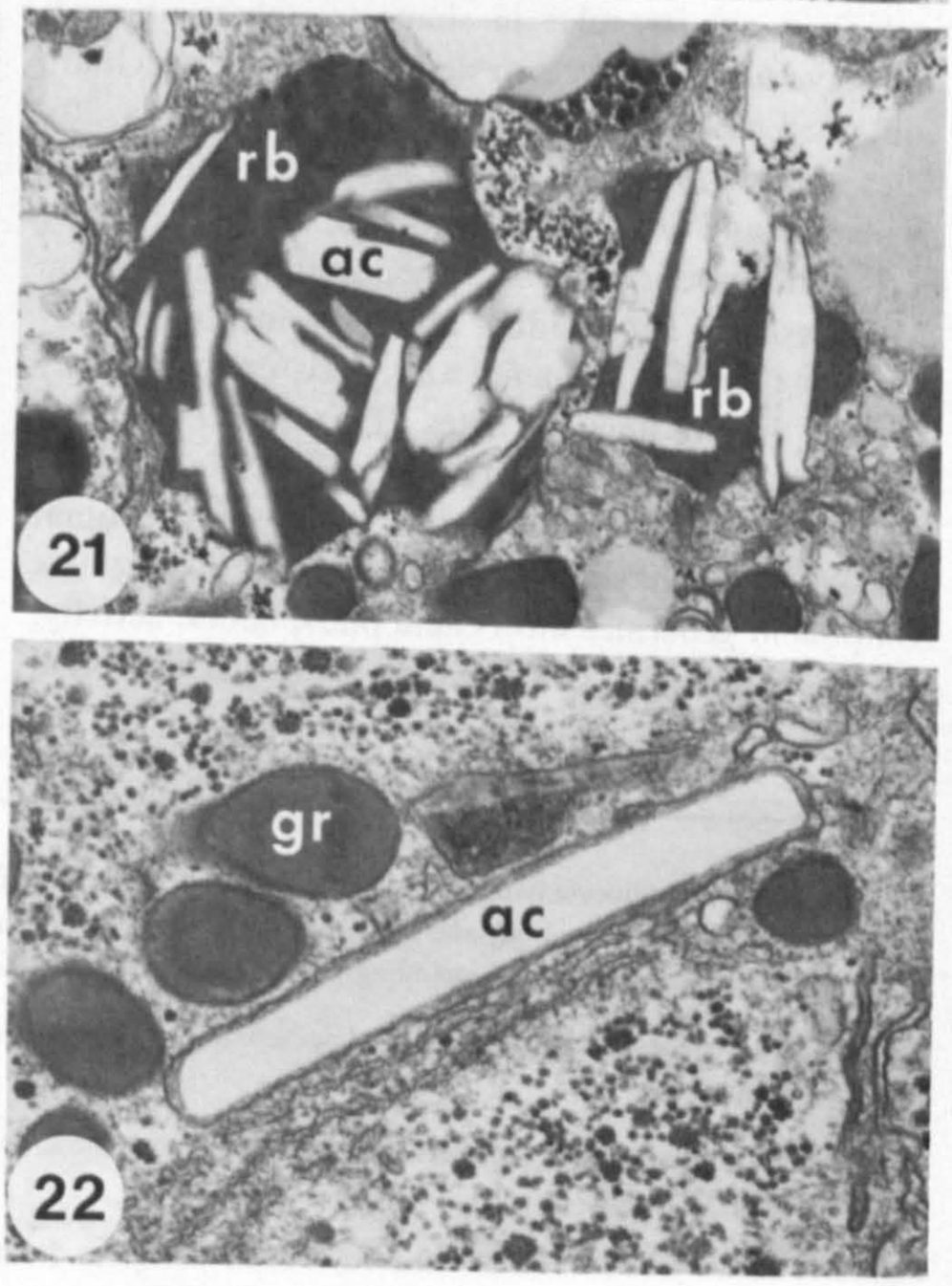
17



19



20



21

22

Figs. 16–22

between three and six narrow cisternae, often surrounded by numbers of small vesicles. The contents of the cisternae usually increase in density from one side of the stack to the other, and the stack is often associated with a single cisterna of endoplasmic reticulum (Fig. 16). The complex may be associated with one or more kinds of granule. Often these granules have a mottled appearance (Figs. 16 and 17), or they may resemble smaller versions of the characeristic amoebocyte dense granules described above (Fig. 18). It seems likely that these granules are Golgi derived. Often the Golgi apparatus is located in the vicinity of a centriole (Fig. 18) but the significance of this apparent association is not clear.

Sometimes amoebocytes are seen containing typical coelenterate basal-body-rootlet complexes. These consist of two centrioles aligned at right angles to each other and connected to a striated rootlet (Fig. 19). Similar complexes have been described giving rise to flagella in a range of coelenterate cell types (CHAPMAN 1974). No flagella have been observed arising from these complexes in *A. fragacea* amoebocytes, and their function is uncertain.

Amoebocytes often contain what have been termed residual body-like inclusions (CHAPMAN 1974). These inclusions are irregular in shape, membrane-bound, and have a highly electron dense matrix in which are contained characteristic electron lucent cores (Fig. 20). These clear cores are usually straight sided and angular in shape, and may once have contained a possibly crystalline material which has been lost during fixation or processing. The residual body-like inclusions may reach 1–2 μm in diameter and may contain many electron lucent angular cores (Fig. 21). The dense matrix may be heterogeneous and contain membranous remnants or whorls. Rarely, they may contain a single, needle-like angular core (Fig. 22). The matrix component generally shows acid phosphatase activity, which would tend to confirm their presumed lysosomal origin.

3.1. Phagocytic Activity

Many granular amoebocytes show evidence of phagocytic activity. Within the gonad, phagocytosis was most apparent during gamete breakdown and resorption. Oocytes appear to undergo breakdown at a low level throughout the year, and, during and after the spawning period, breakdown of both oocytes and spermatogenic cysts may occur on a considerable scale. Amoebocytes within the gonad appear to be at least partly responsible for the resorption of the degenerating gametes. During these periods, amoebocytes may be found containing what appear to be phagosomes, which may include yolk granules, large lipid droplets and also membrane-bound portions of ooplasm (Fig. 23). Occasionally, amoebocytes are seen apparently in the process of encircling and engulfing such particles (Fig. 24). Amoebocytes containing gamete debris can be found some distance away from the degenerate gametes, suggesting that they can move away after phagocytosis has occurred. Many amoebocytes contain various dense bodies, inclusions and residual body-like structures which may well be lysosomal in origin and may be indicative of earlier phagocytic activity. No inclusions obviously resembling bacteria or other microorganisms were found in any of the cells studied.

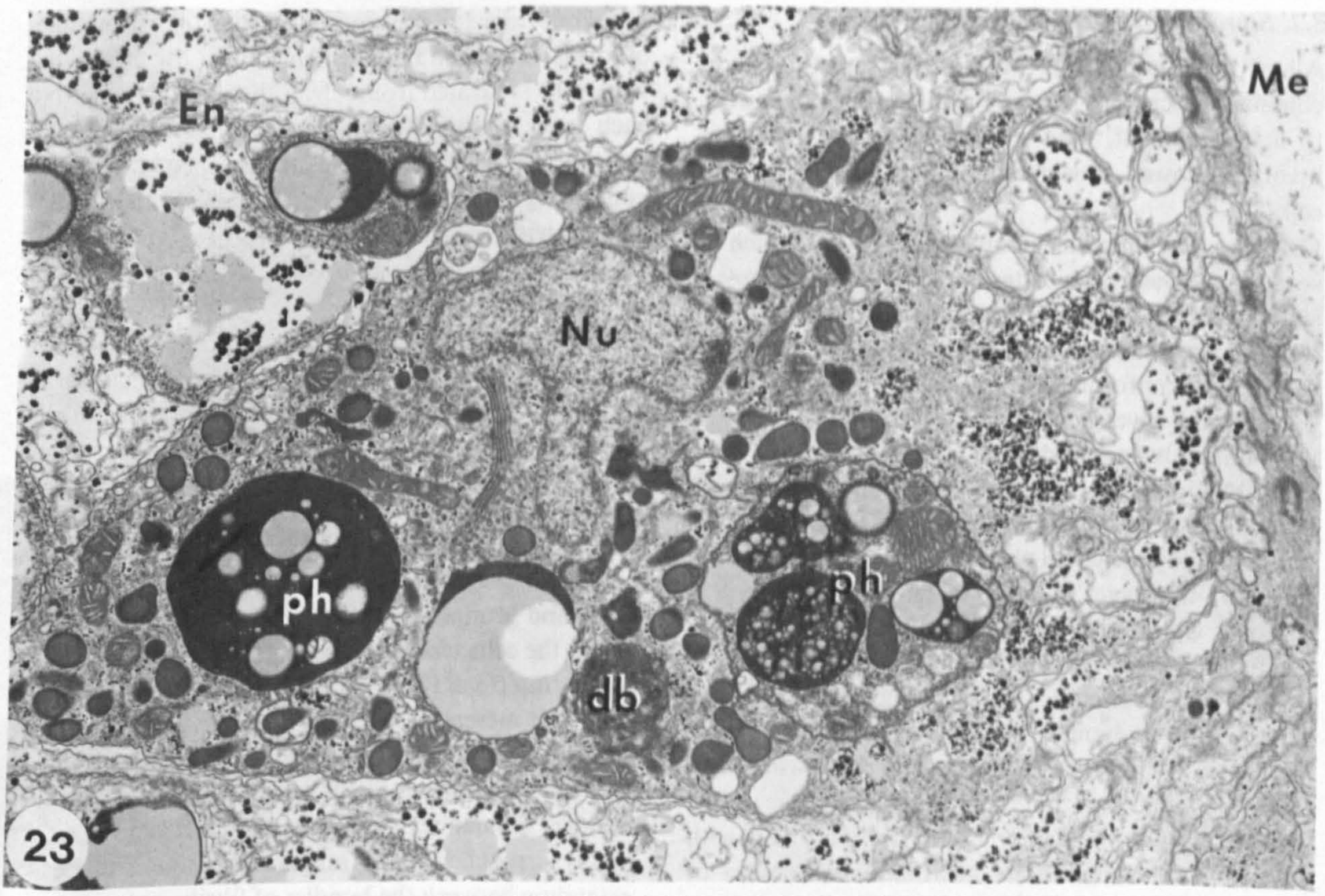
The cytoplasm of many amoebocytes contains large, rounded, membrane-bound vacuoles (Figs. 2 and 4). Usually these vacuoles appear empty, but they sometimes contain material similar to that surrounding the cell. They are most common in cells in or near the mesogloea, and are rare in regularly-shaped cells in the epithelia (Fig. 25). Amoebocyte processes in the mesogloea are often highly branched and may contain many of these vacuoles (Fig. 26). They are sometimes seen to open to the outside, and it seems possible that they arise by the fusion or anastomosis of slender cytoplasmic extensions. This process may result in the interiorization of droplets of the external medium.

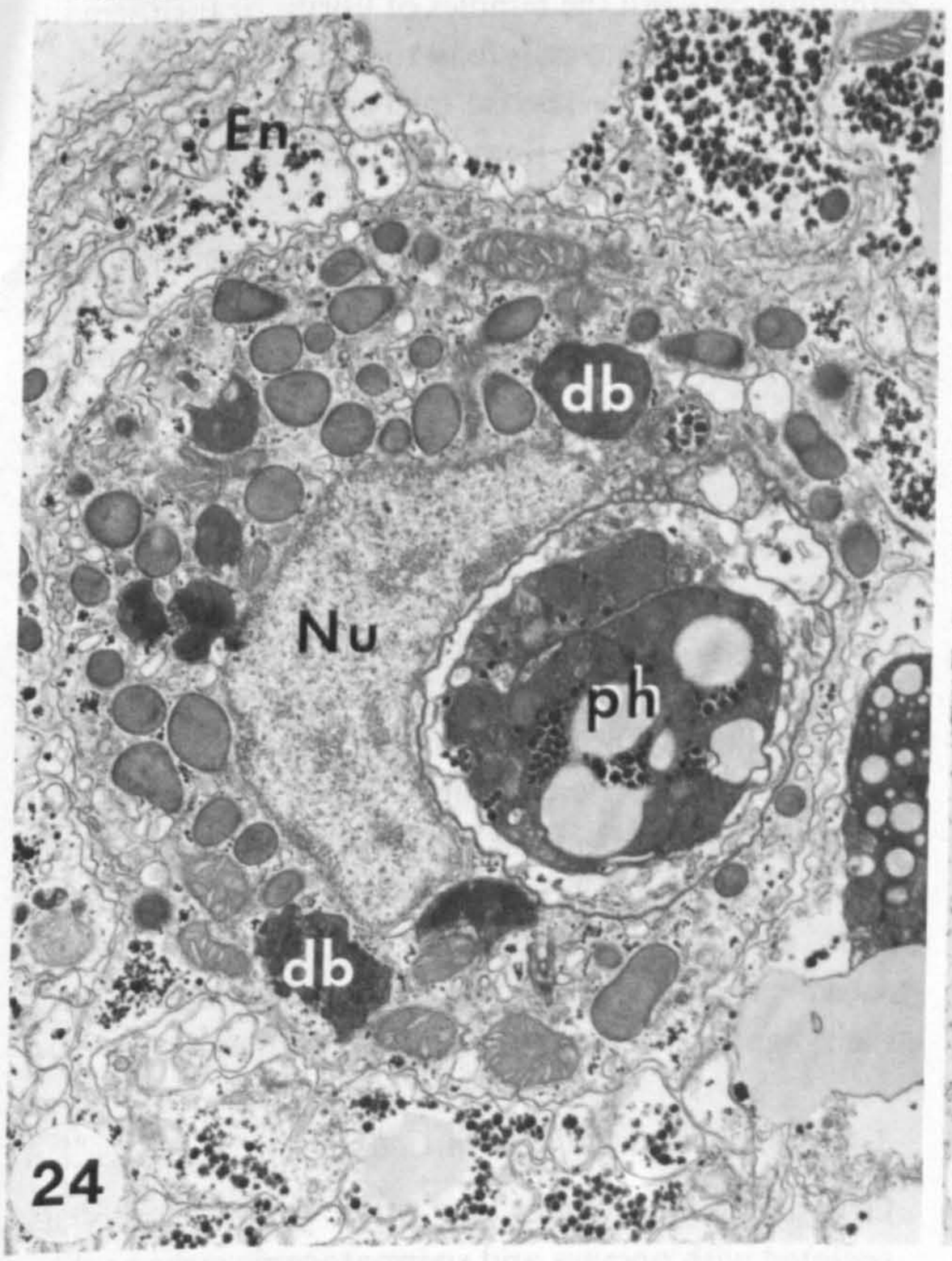
Fig. 23. A large amoebocyte lying in the endoderm, close to the mesogloea, during a period of extensive oocyte breakdown. It contains several inclusions, including a dense body and two phagosomes. The phagosome on the left contains a large yolk granule, while that on the right contains a fragment of oocyte cytoplasm, including yolk granules and mitochondria. Mag. × 12,000

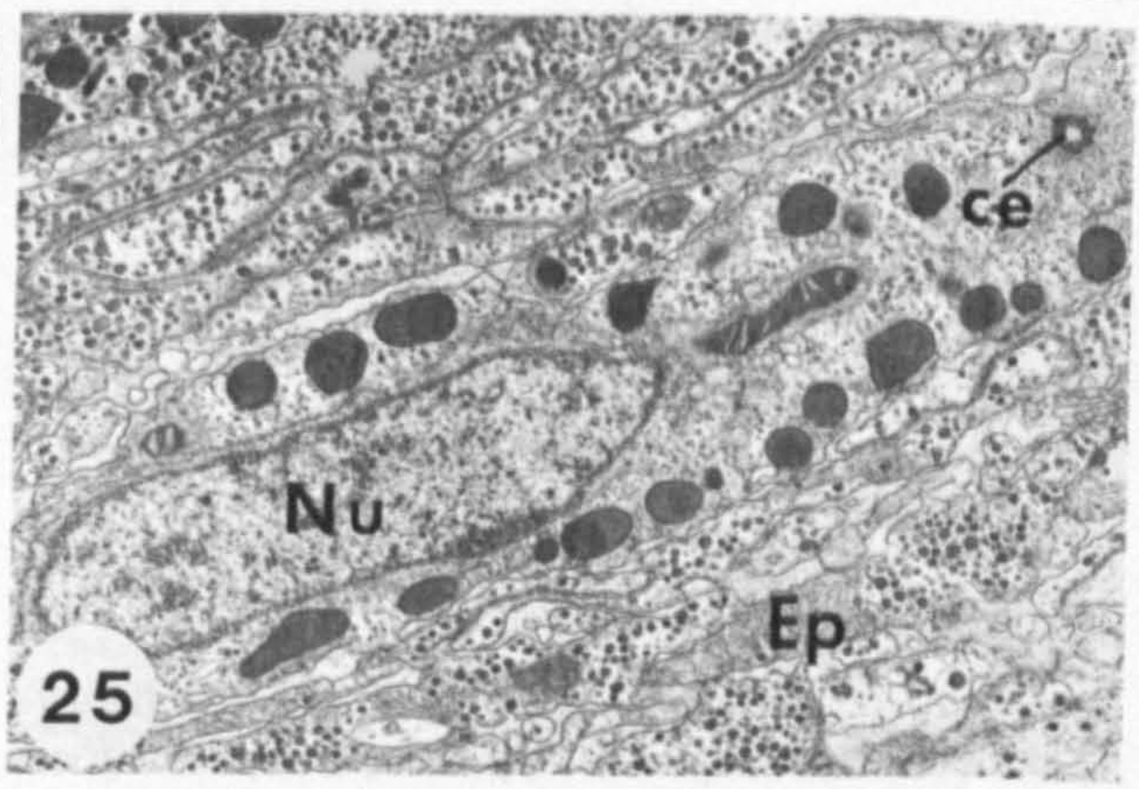
Fig. 24. An amoebocyte, containing several dense bodies, apparently in the process of engulfing a large phagosome containing oocyte debris. Mag. × 13,000

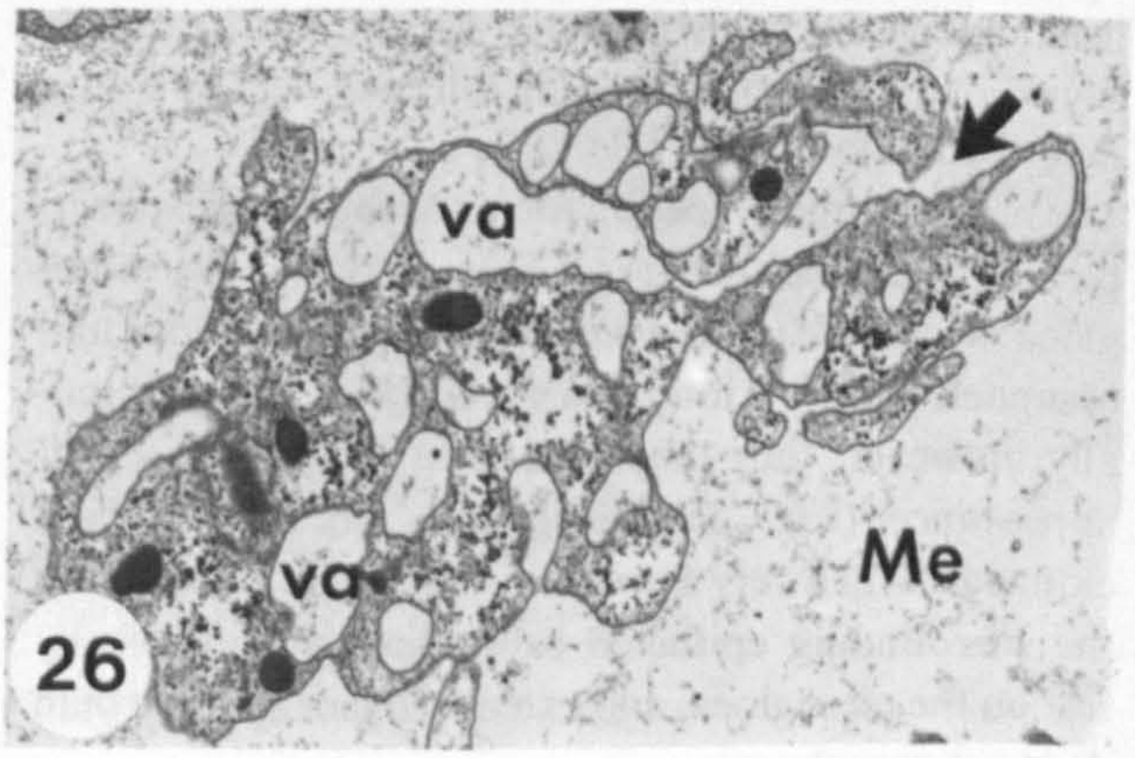
Fig. 25. An amoebocyte lying parallel to the epithelial cells of the endoderm. It has a very smooth outline, with no processes, and it contains no vacuoles. Mag. × 11,000

Fig. 26. A slender process from an amoebocyte in the mesogloea. It is branched and irregular, and contains numerous vacuoles, some of which connect to the surface of the cell (arrowed). Mag. × 9,000









Figs. 23–26

3.2. Storage of Reserve Materials

All of the amoebocytes observed during this study contained reserve material in the form of glycogen and, to a lesser extent, lipid droplets, but the quantities involved showed considerable variation from cell to cell.

Glycogen was found as cytoplasmic deposits which in some cases were quite extensive and could occupy a significant proportion of the cell. The glycogen in these deposits occurs both as the characteristic rosettes of the α-glycogen conformation and as individual smaller particles of β-glycogen. It is noticeable that rosettes are less numerous and tend to be smaller in the amoebocytes than in the glycogen deposits of the epithelial cell bases. Many amoebocyte glycogen deposits contain large, roughly spherical clear areas, often surrounded by a very thin layer of dense material (Fig. 2).

In some cells, large quantities of glycogen may be located in peripheral cytoplasmic lobes (Fig. 27). The lobes appear as swellings on the surface of the cell, and may be 5 μm in diameter. They often connect with the rest of the cell by only a slender neck of cytoplasm. Each lobe is bounded by a thin rim of cytoplasm beneath the membrane, but the center of the lobe is filled with glycogen. Other inclusions, such as mitochondria or dense granules, are rarely found in these lobes. Less commonly, amoebocytes may display one or sometimes two larger glycogen-filled lobes (Fig. 28), which may occupy up to half the apparent volume of the cell.

Most amoebocytes contain a small number of lipid droplets. These are usually homogeneous, of low electron density, and range from 0.4 to 1 μm in diameter. Occasionally, cells containing much larger numbers of lipid droplets are found (Fig. 29).

3.3. Variation in Appearance

Granular amoebocytes in *A. fragacea* are highly variable in their shape and appearance, depending, at least in part, on the situation in which they are found. Some of these variants from different locations are described below.

The most common location for amoebocytes appears to be among the epithelial cell bases, close to the mesogloea. Most cells in this location tend to be rounded and compact, with few long extensions (Fig. 2). Occasionally, however, they may take on a more "epithelial" appearance (Fig. 25). Such cells are slender and elongate, with a very smooth outline, and lie parallel to the surrounding epithelial cells. Basally they usually rest on the mesogloea, and extend further apically than do most amoebocytes.

Amoebocytes within the mesogloea can take on a range of forms. Other cell types which contact the mesogloea tend to be separated from it by a thin basal lamina. No such layer is found around amoebocytes, however. Amoebocytes in areas of relatively open mesogloea, containing few collagen fibrils, tend to have an irregular outline with long cytoplasmic processes. These processes may branch, and often contain numerous vacuoles (Fig. 26).

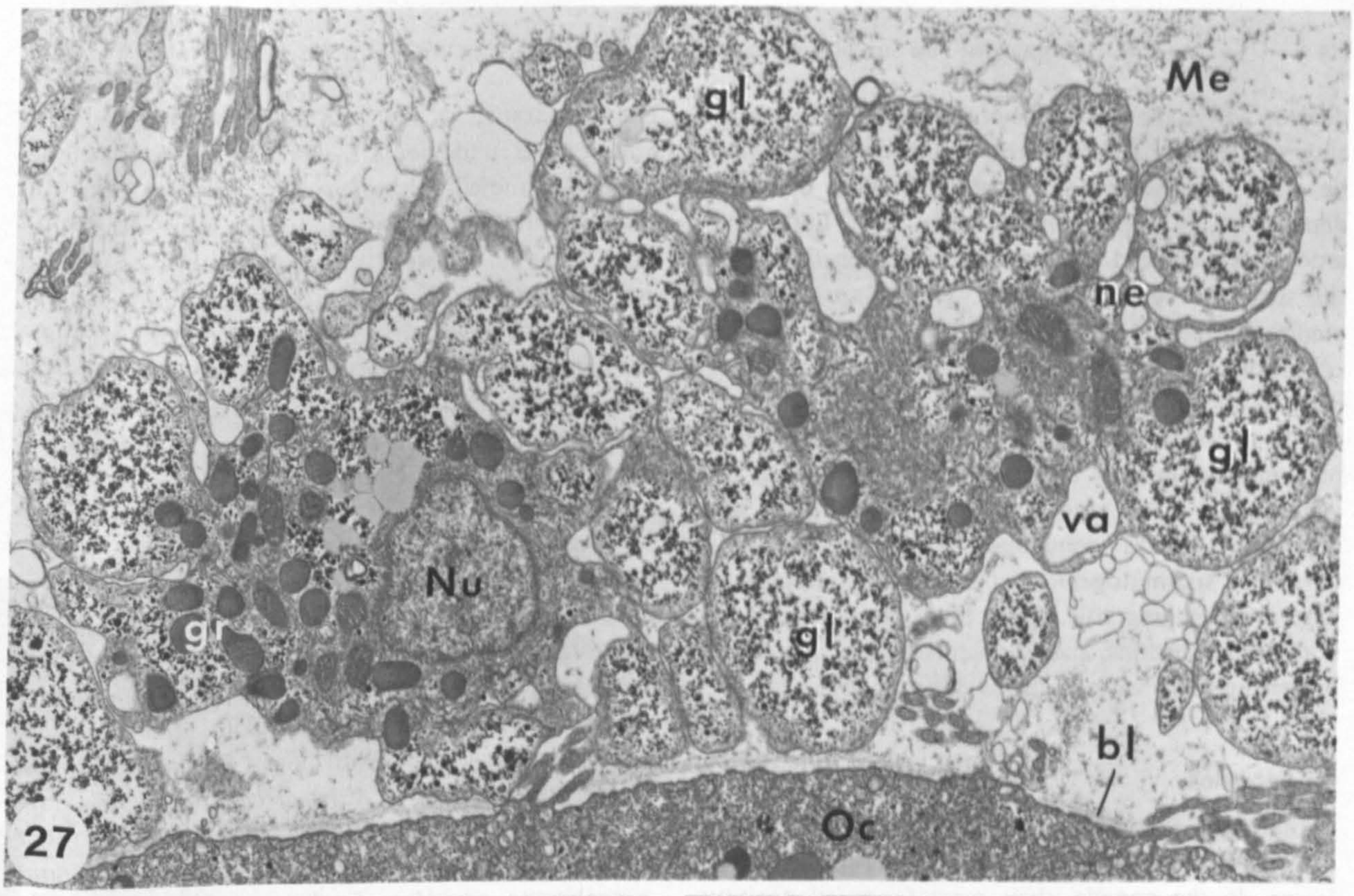
Fig. 30 shows an amoebocyte in an area of mesogloea contain numerous randomly arranged collagen fibrils. The cell has a "pleated" appearance, common in this type of mesogloea, in which the cell bears numerous cytoplasmic extensions which are tightly folded and packed together. Thus the cell appears compact in overall shape, but the outline of the cell membrane is highly complex. Sometimes two or more amoebocytes are found grouped together in this type of mesogloea. Again the cells tend to be irregular in outline, but may closely interlock (Fig. 31).

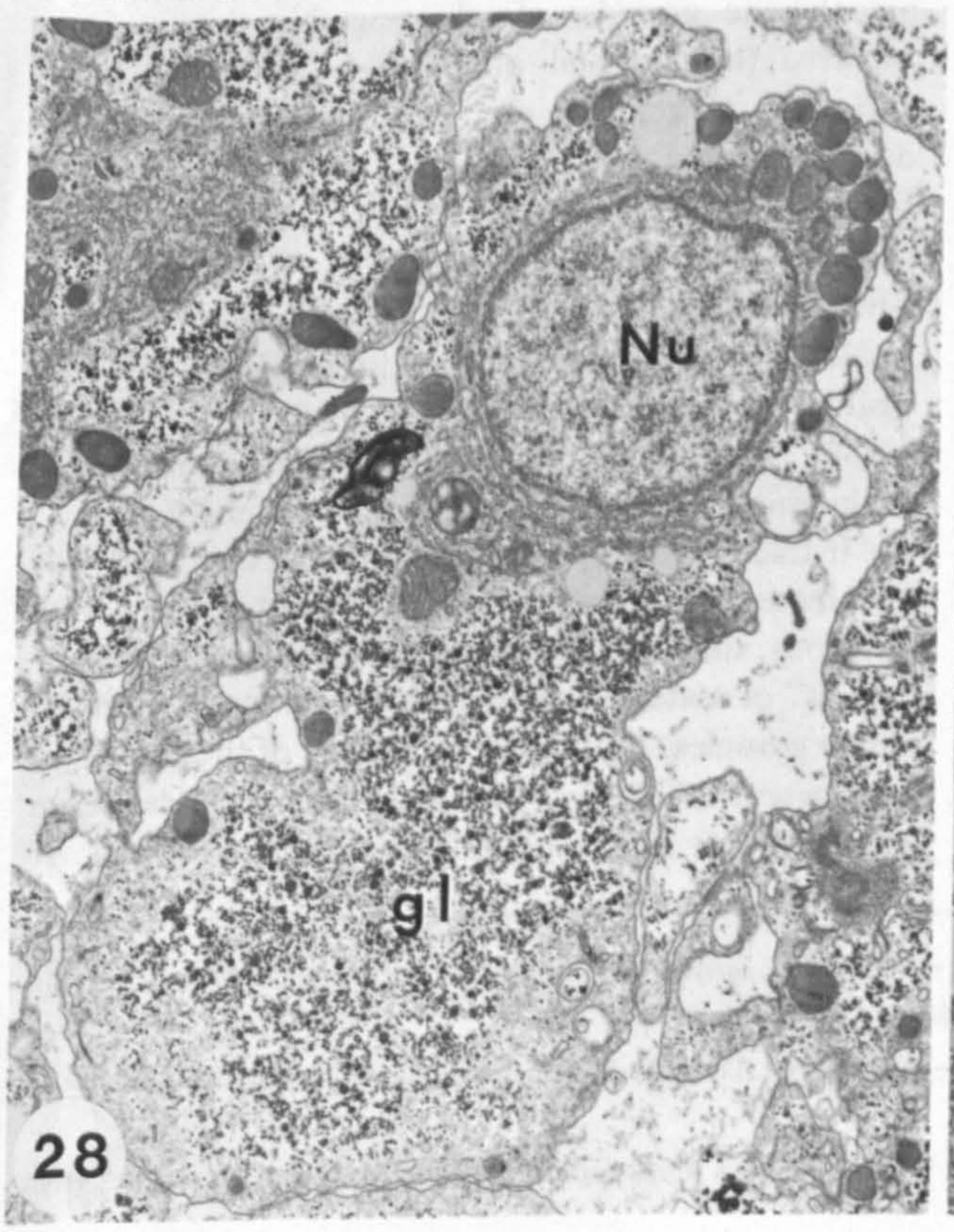
Near the mesenteric retractor muscle, the mesogloea usually has a more ordered structure, with large numbers of collagen fibrils packed into tight, parallel bundles. Amoebocytes in these regions tend to be either highly irregular, with numerous slender processes extending between the bundles of fibrils, or fusiform in shape. This fusiform cells tend to have elongate nuclei, and may extend for several micrometres in a roughly straight line (Fig. 32). Sometimes several such cells lie orientated in a similar direction.

Occasionally the membrane around amoebocyte processes in regions of closely fibrous mesogloea appears to be disrupted. This may allow glycogen and other contents of the process to escape into the surrounding mesogloea (Fig. 33). Sometimes these processes contain granules which are larger and less dense than the typical amoebocyte granules, and which might represent these granules in a less condensed form (Fig. 34). These more expanded granules may be associated with small bundles of fibrils of similar appearance to the mesogloeal collagen fibrils. They could represent a stage in the formation of new collagen fibrils from precursor material contained in the less dense granules, or could be involved in the breakdown of existing fibrils, perhaps during re-modelling of the mesogloea. Such activities were only encountered rarely, however, and their significance is uncertain (see Discussion).

3.4. Associations with Germ Cells

Granular amoebocytes are often found closely associated with oocytes and spermatogenic cysts as these





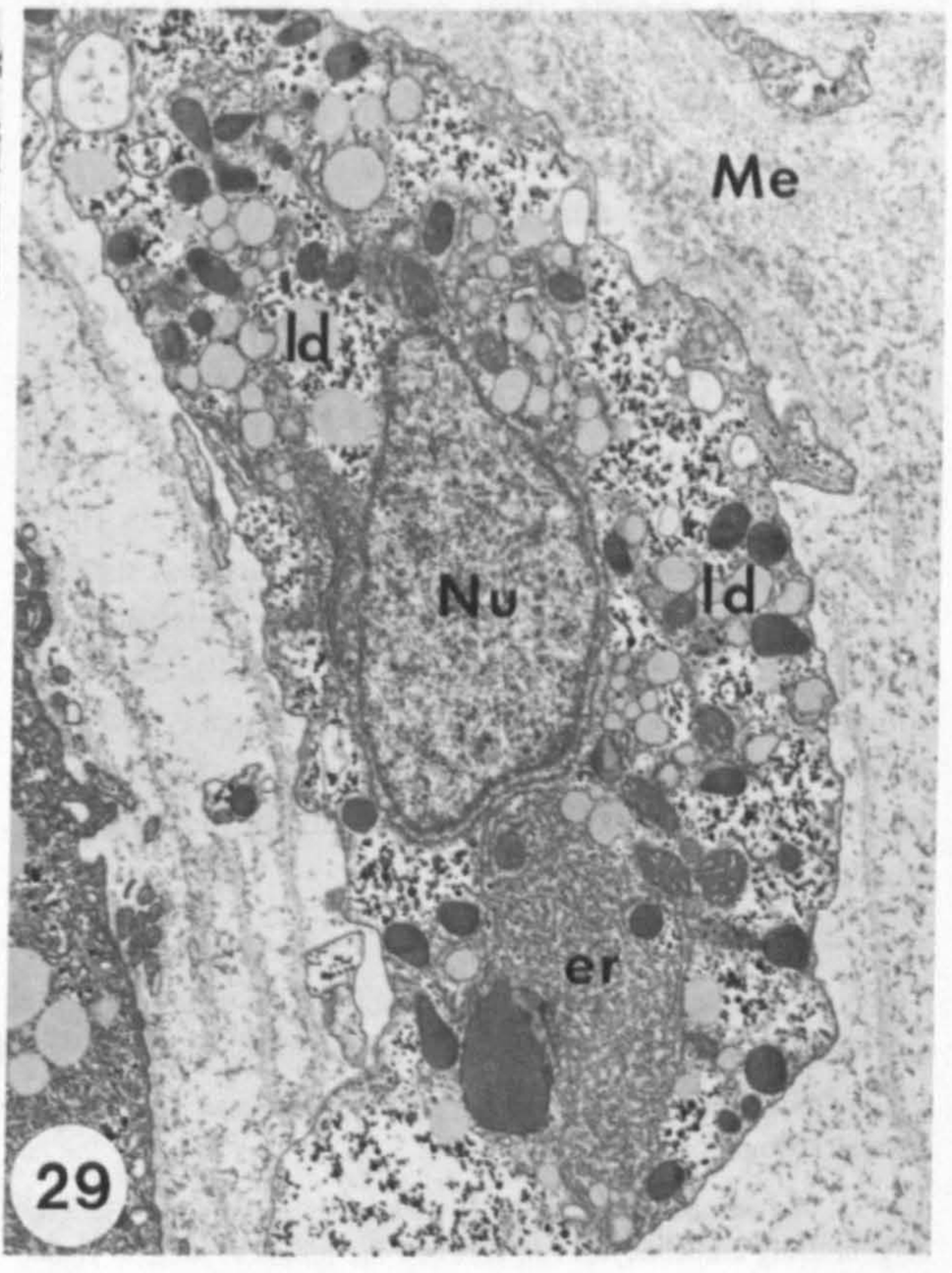


Fig. 27. Amoebocyte in the mesogloea close to a developing oocyte. The cell bears several peripheral lobes filled with glycogen, some of which are only connected by a slender neck of cytoplasm. The lobes are virtually devoid of organelles. Mag. × 10,000

Fig. 28. An amoebocyte with a single, large lobe filled with glycogen. Mag. × 12,000

Fig. 29. An amoebocyte in the mesogloea, containing an unusually large number of small lipid droplets. Mag. × 9,000

develop within the mesogloea of the gonad. In the early stages of gamete development, rounded amoebocytes lie close up against the oocytes or cysts, often in depressions in their surfaces (Fig. 35). Later the amoebocytes spread out and become flattened around the surface (Figs. 36 and 37). They always lie immediately outside the basal lamina which covers the oocyte or cyst, but at no time do they form a continuous layer. In the female, closer contact between oocyte and amoebocyte may be provided by the cytospines, which may project through the basal lamina into deep invaginations in the amoebocyte cytoplasm (Fig. 36). Amoebocytes are sometimes found inside well-developed spermatogenic cysts, where they appear to play a scavenging role. They often contain residual body-like inclusions, and fragments of what appear to be germ cells in various stages of breakdown.

4. Discussion

In *Actinia fragacea*, granular amoebocytes are found both in the mesogloea and among the epithelial cell layers. In the gonads, their most favoured position appears to be among the tangled basal processes of the endodermal cells. MINASIAN (1980) reports a similar situation for the sea anemone *Haliplanella luciae*. It would appear that amoebocytes can move freely between endoderm and mesogloea. With the light microscope, however, even when using 1 µm plastic sections, it is difficult to distinguish amoebocytes among the epithelial cell layers, but they are easily recognized in the mesogloea. This may help to explain why much of the early literature (*e.g.*, HYMAN 1940) suggests that amoebocytes have a predominantly mesogloeal distribution. CHAPMAN (1974) could only distinguish amoebocytes in the endoderm of *Aurelia* at the electron microscope level. PATTERSON and LANDOLT (1979), despite working with the electron microscope, do not mention amoebocytes occurring in the epithelial layers of *Anthopleura elegantissima*.

The appearance of the granular amoebocytes in *A. fragacea* was fundamentally similar in all regions of the anemone studied, although they tend to take on characteristic conformations in specific situations. They broadly resemble previously published micrographs of amoebocytes from other coelenterate species. The appearance of the characteristic dense granules does appear to vary in different species, however. The granules shown by GRIMSTONE *et al.* (1958) from amoebocytes of *Metridium senile* seem to be bounded by a loosely fitting membrane. Similarly, those shown by CHAPMAN (1974) in amoebocytes from both the scyphozoan *Aurelia aurita* and the sea anemone *Calliactis parasitica* appear to have a clear halo between the dense contents of the granule and the surrounding membrane, such that he refers to them as "areolar granules". VAN PRAET and DOUMENC (1975) describe amoebocytes or macrophages from *Actinia equina* which contain granules with closely fitting membranes. In the present study of *A. fragacea* amoebocytes only a tiny proportion of granules had anything other than closely fitting membranes. *Cereus pedunculatus* amoebocyte granules closely resemble those of *A. fragacea* (unpublished observation). Whether these variations reflect true species differences or merely different preparative procedures is unclear.

During the present study, the amoebocyte dense granules did not give a convincingly positive reaction when tested for acid phosphatase enzyme activity, although lysosomal derivatives in epithelial cells appeared strongly positive. WATSON and MARISCAL (1983), however, have reported acid phosphatase activity in granules and Golgi complexes of amoebocytes in the tentacles of *Haliplanella luciae*, and concluded that the dense granules were primary lysosomes.

Previous authors have not reported the existence of "cores" of material of different appearance within the dense granules. An interesting feature of these cores in *A. fragacea* is that they may appear more or less dense than the rest of the granule in different sections. A

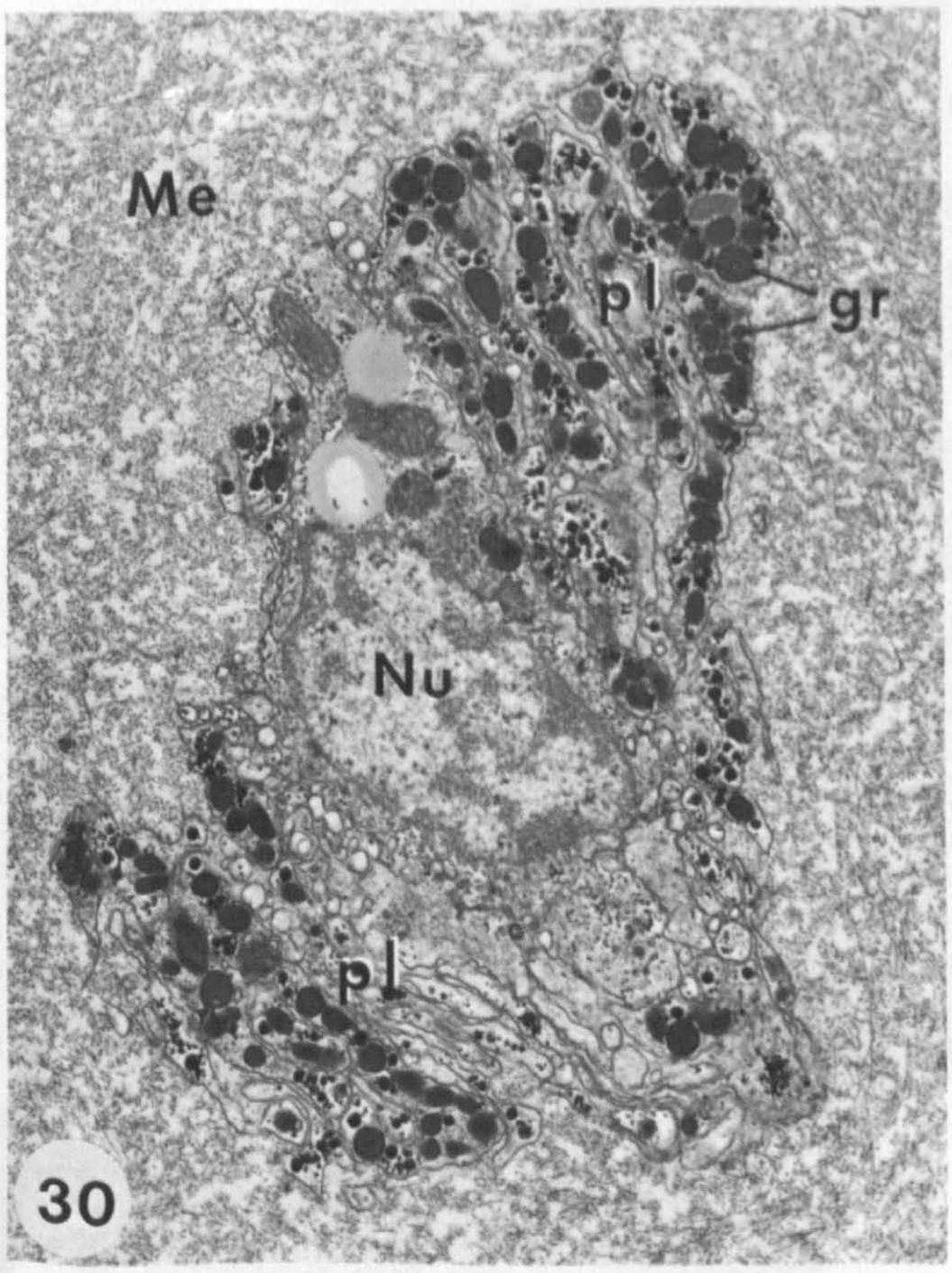
Fig. 30. Amoebocyte in an area of the mesogloea containing numerous randomly arranged collagen fibrils. The cell contains "pleated" regions, consisting of fine cytoplasmic processes tightly folded and packed together. Mag. × 13,000

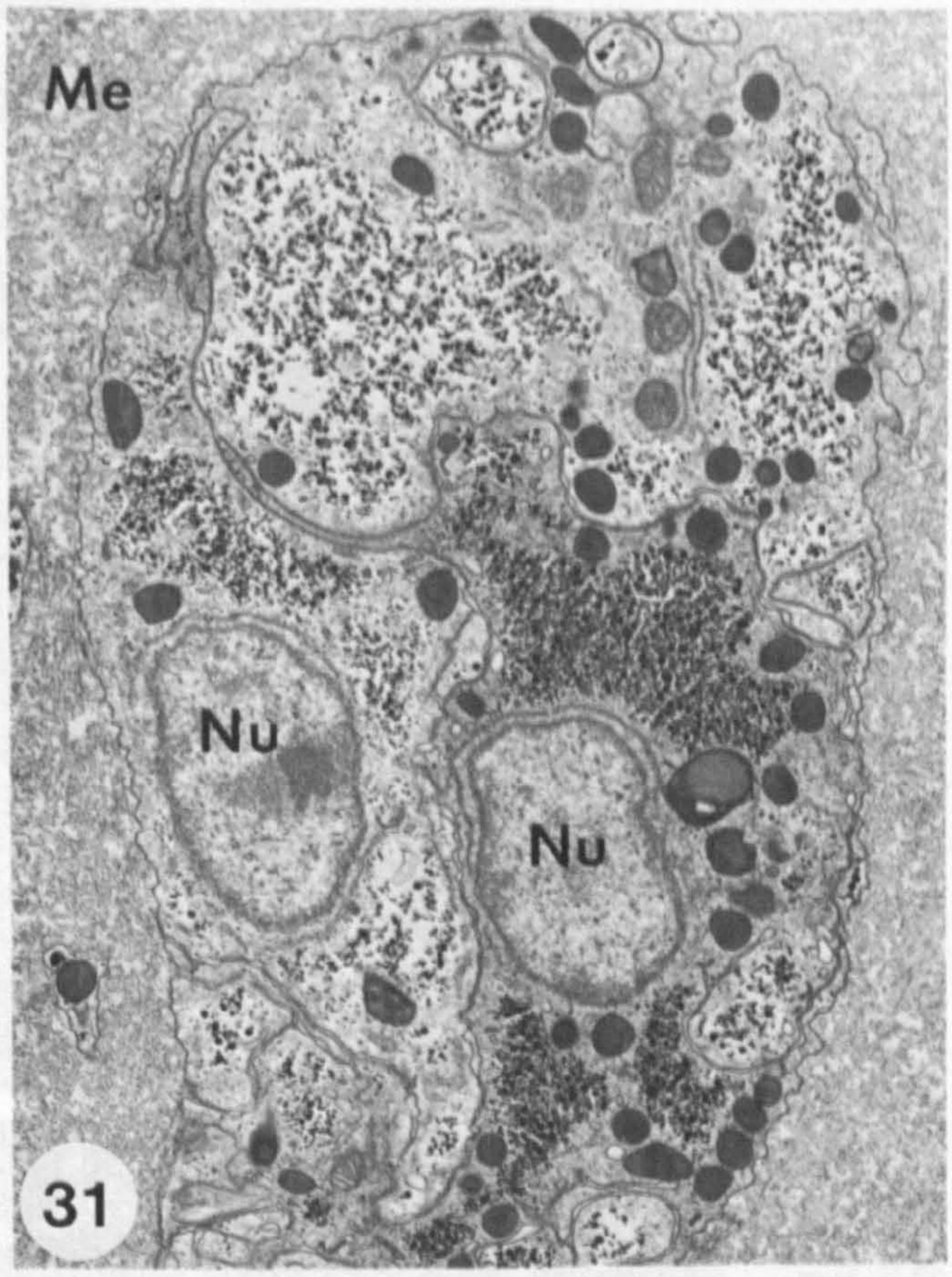
Fig. 31. Two amoebocytes grouped together in an area of randomly fibrous mesogloea. The two cells are irregular in outline, but closely interlock together. Mag. × 10,000

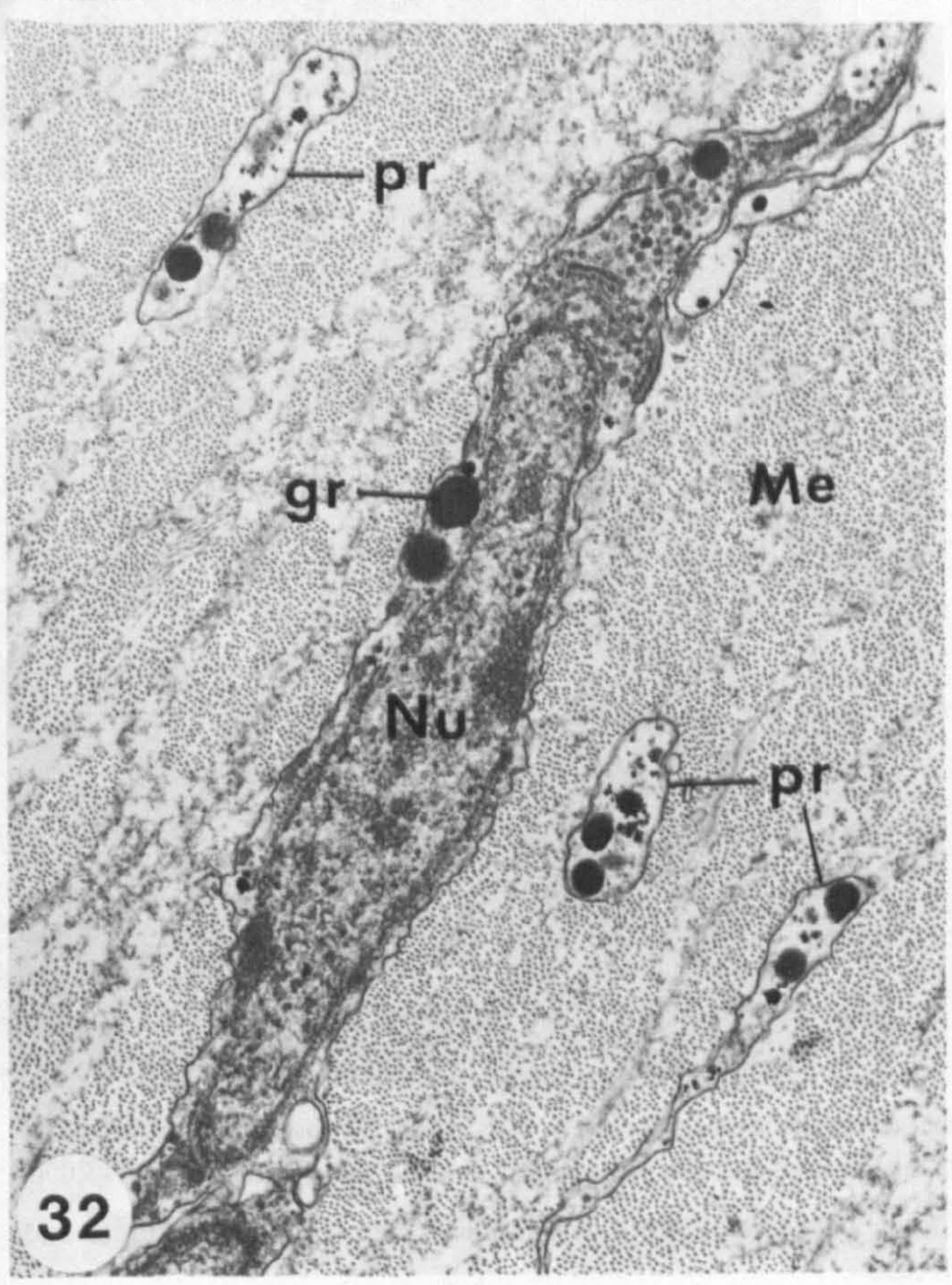
Fig. 32. A fusiform amoebocyte in mesogloea containing numerous parallel collagen fibrils arranged in bundles. Slender processes from neighbouring amoebocytes run between the bundles. Mag. × 18,000

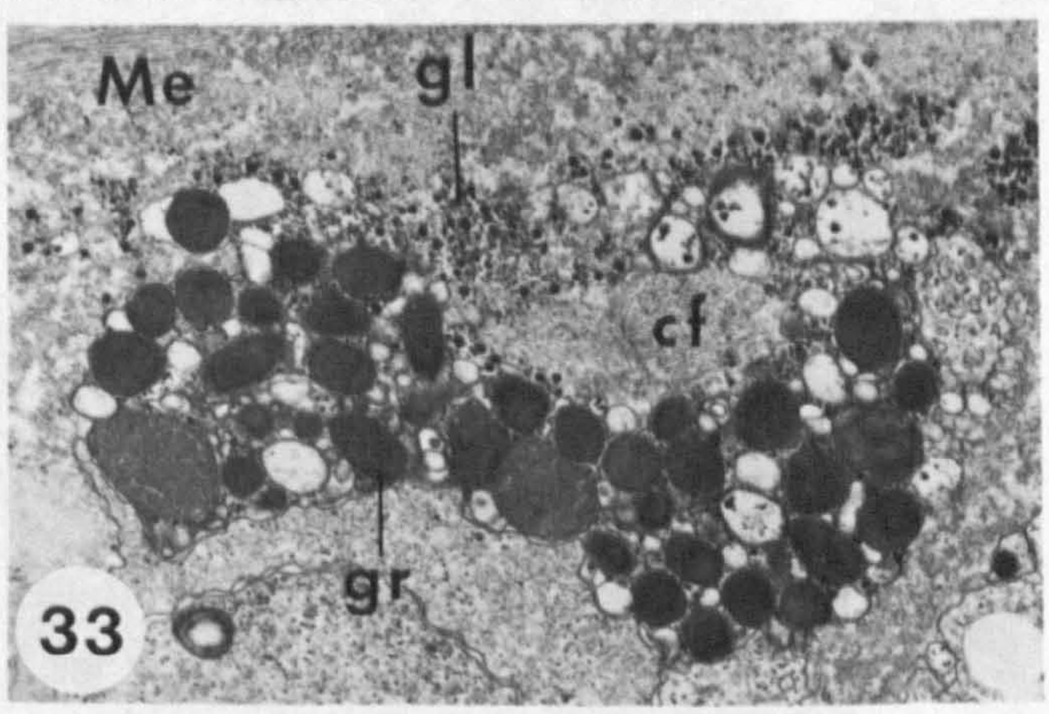
Fig. 33. An amoebocyte process in dense mesogloea. The membrane around the process is disrupted, and glycogen has been released into the mesogloea. The process surrounds two small bundles of collagen fibrils. Mag. × 13,000

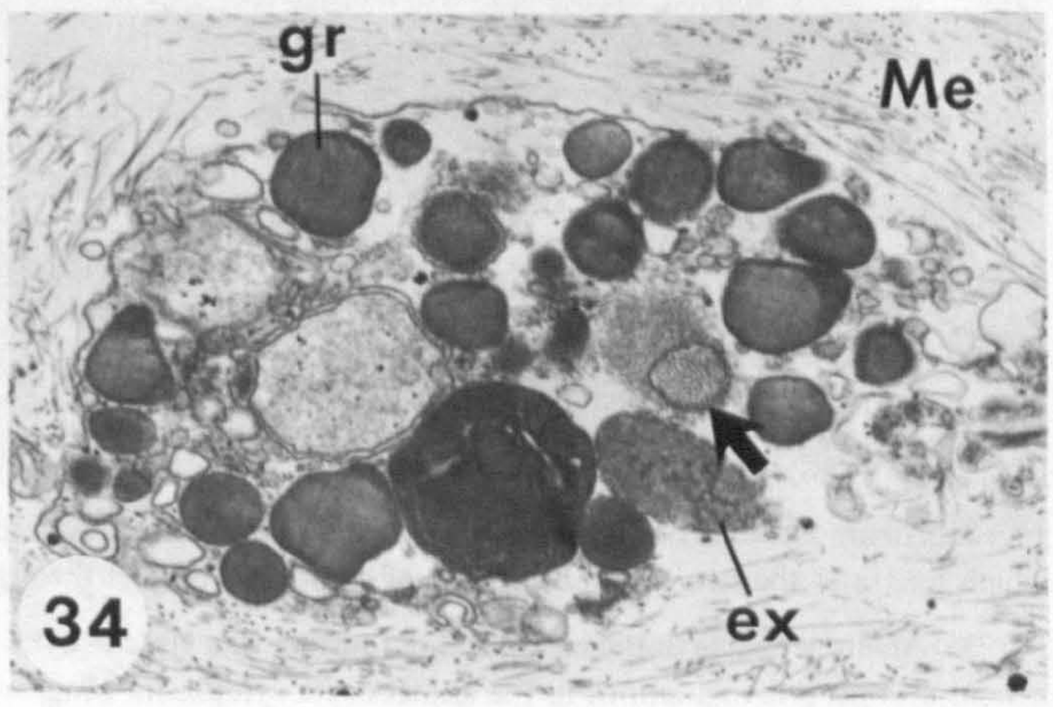
Fig. 34. Another process with a disrupted membrane. It contains two granules which may be expanded forms of the dense granules, one of which is associated with a small group of fibrils (arrowed). Mag. × 20,000


Me
pl
gr
Nu
pl
30

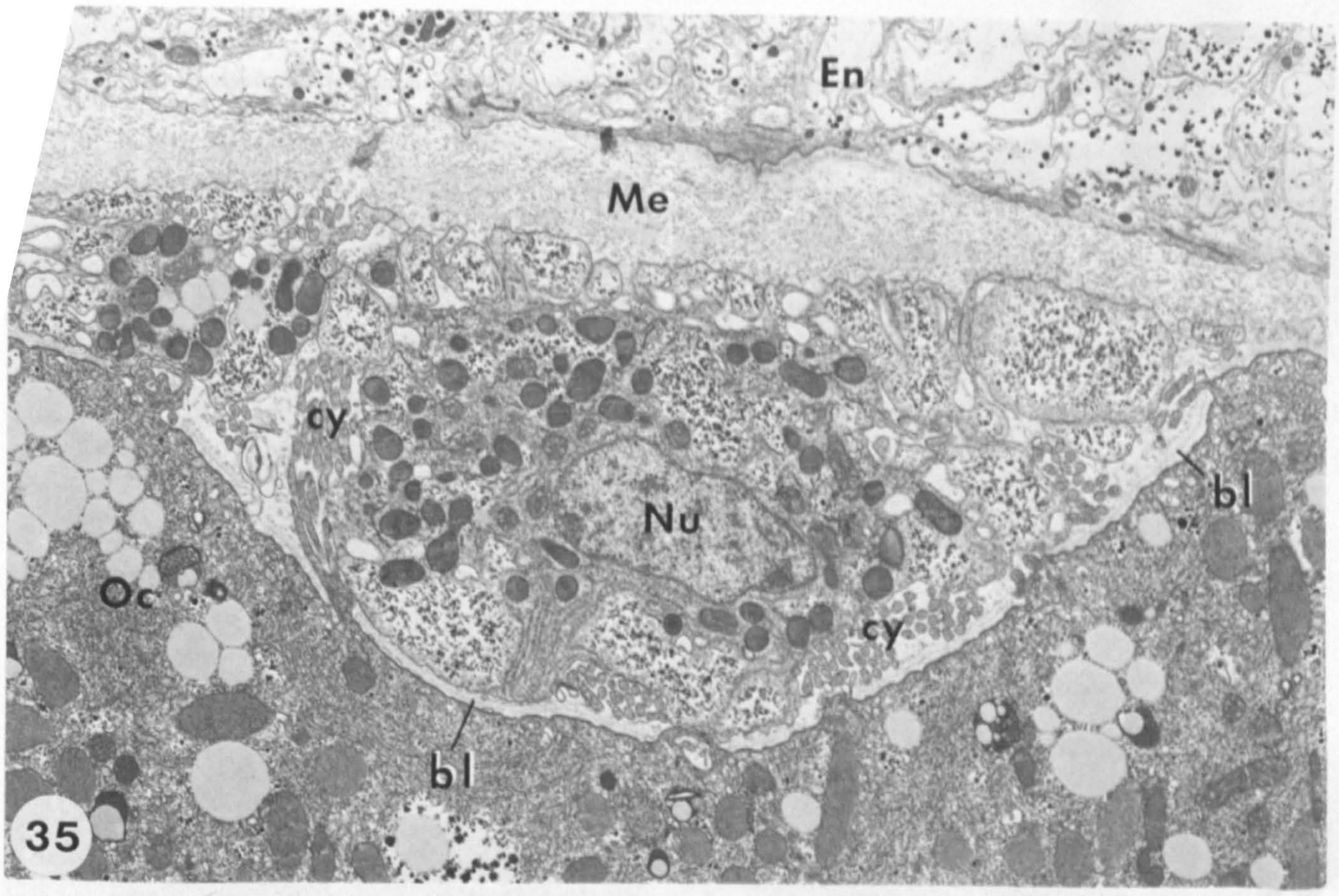

Me
Nu
Nu
31

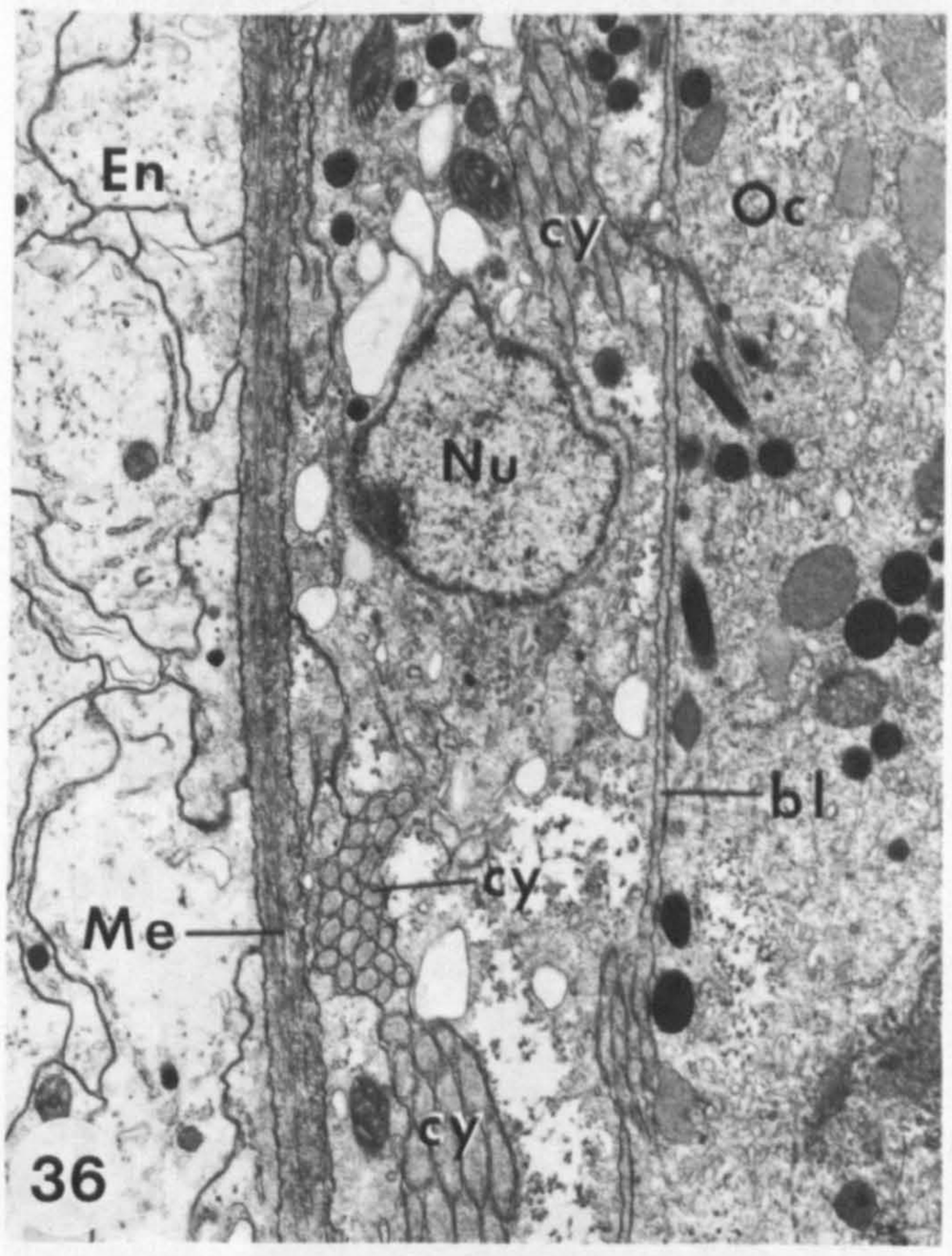

pr
gr
Me
Nu
pr
32


Me
gl
cf
gr
33


gr
Me
ex
34

Figs. 30–34





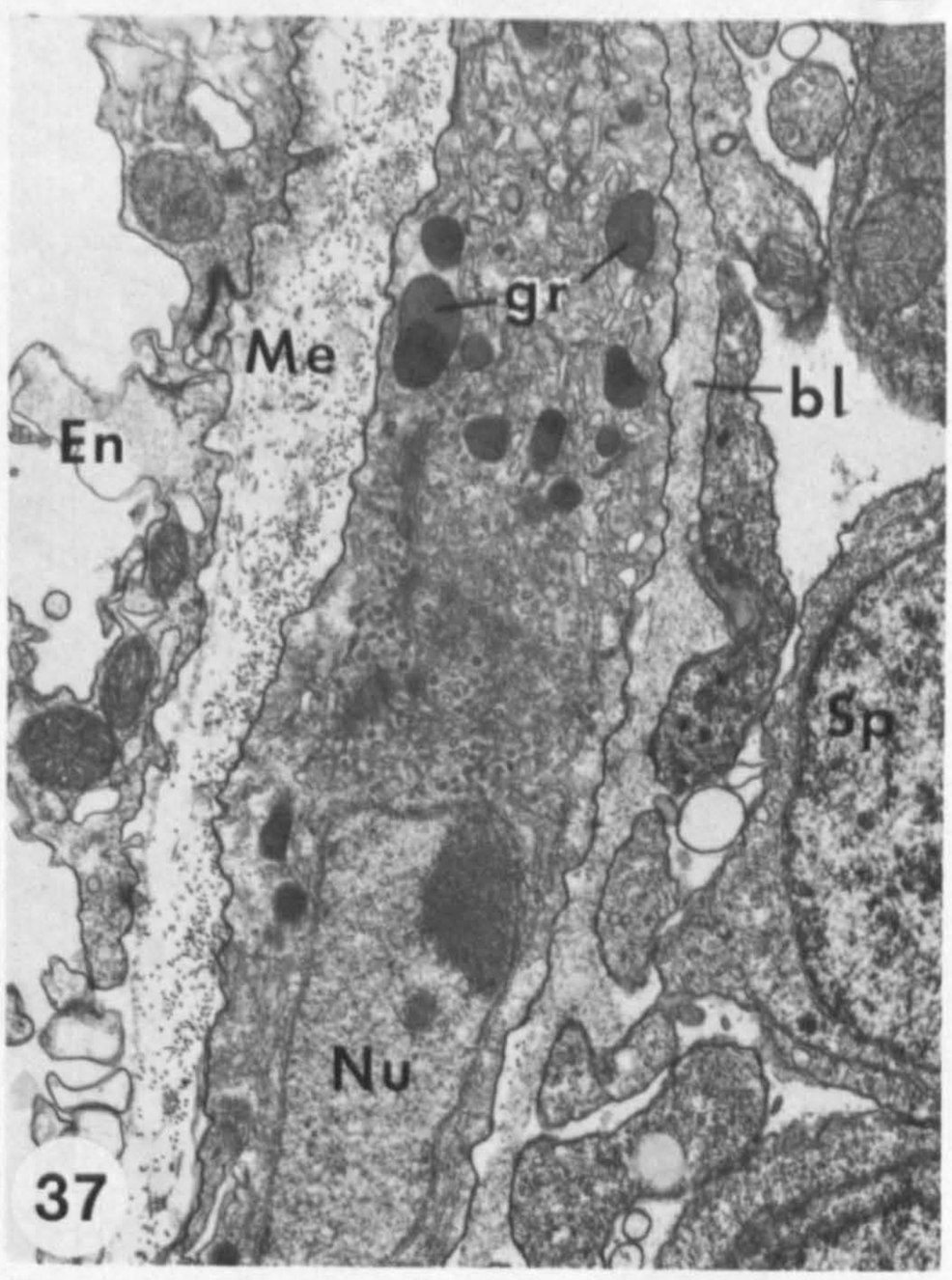


Fig. 35. An amoebocyte lying in a depression in the surface of a developing oocyte in the mesogloea. The oocyte is surrounded by a basal lamina which separates the two cells, but groups of cytospines project through this and contact the amoebocyte membranes closely. Mag. × 8,000

Fig. 36. An amoebocyte flattened around the surface of a large oocyte. Closely packed groups of cytospines pass through the basal lamina and extend into deep invaginations in the amoebocyte cytoplasm. Mag. × 10,000

Fig. 37. An amoebocyte flattened around the surface of a developing spermatogenic cyst. The basal lamina separates it from the spermatogonia at the periphery of the cyst. Mag. × 16,000

possible explanation is that the core may have different sectioning properties from the rest of the granule, and so tends to be cut at a different thickness. Whether this is thinner or thicker than the surrounding granule may vary from section to section. Previous authors also make no mention of membranous elements or vesicular regions within amoebocyte granules from other species. However, in *A. fragacea* these features were found in only a small proportion of the granules examined.

The dense bodies containing angular electron lucent inclusions found in *A. fragacea* amoebocytes have been described from a number of coelenterate cell types. CHAPMAN (1974) reports their occurrence in both ecto- and endodermal epithelial cells in *Aurelia*, and refers to them as residual body-like inclusions, the term used in the present study. SPANGENBERG and BECK (1966) found similar structures in *Aurelia* statoliths. TARDENT and SCHMID (1973) found them in *Coryne* mechanoreceptor accessory cells and termed them crystalline vacuoles. They suggested that these vacuoles could act as primitive mechanoreceptors, but might merely be residual bodies. They occur in many cell types in *Actinia fragacea* (unpublished observation), and are found in male germ cells in *A. equina* (LARKMAN and CARTER 1980). In *A. fragacea* they show acid phosphatase activity, which would tend to confirm their lysosomal origin. TIFFON and HUGON (1977) also demonstrated acid phosphatase activity in similar inclusions in *Pachycerianthus fimbriatus*.

The irregular bodies found in *A. fragacea* amoebocytes are of unknown significance. Their appearance is highly variable, but similar inclusions have been found in absorptive endodermal cells in the gastric filaments, and, less frequently in the apical regions of the gonad epithelial cells. It seems possible that they are involved with intracellular digestion, but acid phosphatase activity has not yet been convincingly demonstrated within them (unpublished observation).

The finding of typical coelenterate basal-body-rootlet complexes in *A. fragacea* amoebocytes is interesting but not unique. VAN PRAET (1978) found ciliary rootlets in amoebocytes from the mesenteries and filaments of *Actinia equina*. KANESHIRO and KARP (1980) reported ciliary rootlets in circulatory coelomocytes of the sea-star *Dermasterias*, and suggest that this might argue that the circulating coelomocytes could be derived from stationary flagellated epithelial cells. In coelenterates, ciliary rootlets seem to occur in a variety of non-epithelial cell types. KESSEL (1968) found basal-body-rootlet complexes in the early oocytes of an unidentified hydrozoan medusa, as did LARKMAN (1981, 1983) for early oocytes of *A. fragacea*. They also occur in the mesogloeal cells of the upper gastric filament in *A. fragacea* (unpublished observation). It may be that most sea anemone cells possess such a complex, which may not always be functional.

PATTERSON and LANDOLT (1979) have investigated the cellular response to injury in the sea anemone *Anthopleura elegantissima*. They found that the mesogloea of the body column of uninjured anemones contained a homogeneous population of amoebocytes. These cells possessed numerous granules, but Golgi complexes and endoplasmic reticulum were absent or poorly developed. After injury, a rapid influx of phagocytes, derived from resident mesogloeal amoebocytes, into the damaged region took place. By 72 hours post-injury, two other cell types were found in the repair zone. One type contained rough endoplasmic reticulum and more mitochondria but fewer granules than the resident amoebocytes. The second type showed a proliferation of the Golgi apparatus and smooth endoplasmic reticulum, and a total lack of specific granules. In the present study, amoebocytes from what were thought to be healthy, uninjured *A. fragacea* individuals usually contained Golgi and rough endoplasmic reticulum, and cells resembling the two other cell types described for *Anthopleura* were often found. The amoebocyte population in *A. fragacea* seemed strongly heterogeneous, but this may in part be due to plane of section effects. Many amoebocytes show a distinctly asymmetrical distribution of organelles and inclusions, such that different section planes can give rise to very different appearances. It is not thought that this heterogeneous amoebocyte population represents several discrete cell types, but rather a continuum of forms shown by a single cell type. However, a fuller understanding of the significance of the various forms might require a subdivision into different cell types at some stage in the future.

Various authors have proposed a range of functions for amoebocytes from a number of anthozoan species. The following discussion is aimed to evaluate how far the findings of the present study are consistent with the various proposed functions.

The granular amoebocytes of *A. fragacea* do appear to be motile. The variability of their shapes, and the frequent finding of pseudopodia and more slender cytoplasmic processes argues that these cells are capable of shape change and amoeboid movement. They appear to be able to move between the bases of the epithelial cells and into and through the mesogloea. PATTERSON and LANDOLT (1979), for *Anthopleura*

elegantissima, noted a local increase in the number of amoebocytes in the vicinity of experimental lesions. They concluded that this increase was brought about by the migration of resident amoebocytes within the mesogloea. However, in *A. fragacea*, a proportion of amoebocytes appear to be less actively motile. Some take on a regular cylindrical shape and lie parallel to the epithelial cells in what would appear to be a stable relationship. Similarly, some amoebocytes found around developing oocytes and testicular cysts enter into a precise and apparently stable relationship with them. Thus it appears that while amoebocytes are capable of movement, they may at least temporarily adopt a more sessile mode of existence.

A. fragacea amoebocytes also seem to be capable of phagocytic activity. Many cells were found containing what appeared to be phagosomes in varying stages of breakdown. Sometimes recognizable material such as fragments of degenerate oocytes or groups of spermatozoa could be discerned within these phagosomes. Occasionally cells were seen apparently in the process of surrounding and engulfing such particles. VAN PRAET (1974) and VAN PRAET and DOUMENC (1975) found that, after oral disk amputation in *Actinia equina*, amoebocytes in the vicinity of the wound became highly phagocytic, engulfing cnidocytes and debris from dedifferentiating epithelial cells. Similarly, POLTEVA (1970) found that amoebocytes became active in phagocytosis during regeneration in *Metridium*. WATSON and MARISCAL (1983) observed phagocytic activity among amoebocytes in intact *Haliplanella* tentacles. However, it should be pointed out that, during gamete breakdown and resorption in *A. fragacea* gonads, the neighbouring endodermal epithelial cells also contained numerous phagosomes with fragments of oocytes or sperm. Given the small size and relative scarcity of amoebocytes compared with these epithelial cells, the proportion of the total intracellular breakdown of gametes carried out by the amoebocytes may be small.

In *Veretillium cynomorium*, the mesogloeal collagen fibrils were often found to be concentrated around the mesogloeal cells (BUISSON and FRANC 1969, FRANC 1970), which led to the suggestion that these cells were involved in the synthesis of the collagen fibrils. Similarly, YOUNG (1974) suggested that mesogloeal amoebocytes might produce collagen fibrils during wound healing in the sea anemone *Calliactis parasitica*. During the present study only the most slender of evidence for the production of collagen fibrils by amoebocytes in the intact anemone was obtained. Very occasionally, small bundles of fibrils were found associated with what might be expanded forms of the typical amoebocyte dense granules. Such a mechanism for collagen deposition would be very different from that observed in higher animals (*e.g.*, PROCKOP *et al.* 1979), but a mechanism of collagen formation from precursors within dense granules has been reported for spongocytes and collencytes in some sponges (GARRONE and POTTU 1973, BERGQUIST 1978). However, GRIMSTONE *et al.* (1958) found no evidence that amoebocytes were involved in the secretion of the mesogloea in *Metridium*. SINGER (1974), using autoradiography, examined the incorporation of tritiated proline (a major precursor of collagen) in the sea anemone *Aiptasia diaphana*. He concluded that the amoebocytes, in spite of their fibroblast-like apppearance, were not active in collagen synthesis, and that this function might be performed by the epithelial cells. However, the amoebocytes do appear to be intimately associated with the mesogloea. As noted by VAN PRAET (1978) for *Actinia equina*, there is no basal lamina separating amoebocytes from the mesogloea, and in the present study amoebocyte processes were often seen extending around bundles of collagen fibrils. Many cells contained vacuoles which themselves contained material similar to the non-fibrous mesogloeal matrix. It may be that amoebocytes are involved in the maintenance or re-modelling of the mesogloea, rather than its synthesis.

Several authors (*e.g.*, VAN PRAET 1974, YOUNG 1974) have suggested that amoebocytes may be involved in the transport and storage of food and waste material. A prime site for the uptake of food particles after preliminary extracellular digestion in the enteron in *A. equina* is thought to be the part of the mesentery close to the gastric filament (VAN PRAET 1976, 1978), and the same would appear to be true for *A. fragacea* (unpublished observation). Amoebocytes are common in this region, and in the present study, many of them contained phagosomes. However, the epithelial cells of this region may contain very large numbers of phagosomes, and it is not clear whether the quantity carried by the ameobocytes would be sufficient to make a significant contribution to their distribution.

In *A. fragacea*, the amoebocytes contain variable quantities of glycogen. In some individual amoebocytes, glycogen may occupy a considerable proportion of the total cell volume, and these cells could contribute to the storage and transport of this important reserve material. VAN DER VYVER (1981) discusses cells which she terms glycocytes wandering in the mesogloea of

actinians. The glycocyte she illustrates from *Cereus pedunculatus* is presumably incorrectly labeled, but would appear to correspond to the granular amoebocytes of the present study. She points out the similarity between these cells and the glycocytes which have been described for marine sponges (BOURY-ESNAULT 1977). These cells, also termed gray cells, contain homogeneous osmiophilic inclusions similar to those found in sea anemone amoebocytes, and quantities of glycogen which vary with the physiological state of the sponge. They are thought to be responsible for the storage of glycogen and its transport to areas of high metabolic activity. Circulatory amoebocytes of some higher invertebrates are also thought to be involved in carbohydrate metabolism, and these cells also may contain numerous electron dense granules as well as glycogen deposits (*e.g.*, JOHNSON *et al.* 1973). In *A. fragacea*, the basal regions of the endodermal epithelial cells contain very extensive deposits of glycogen. The amount of glycogen contained in the amoebocytes can only represent a small fraction of the total, and their contribution to the bulk storage of glycogen must be small. However, they may well contribute to the transport of glycogen and the regulation of carbohydrate levels.

The association between amoebocytes and developing gametes seen in *A. fragacea* does not appear to have been reported previously. In sea anemones, the germ cells arise in the endoderm and then migrate into the mesogloea where the greater part of gamete development takes place. Once within the mesogloea, the gametes are largely isolated from contact with other cells. Close contact is maintained with some endodermal cells over a restricted area known as the trophonema (LARKMAN and CARTER 1982), but otherwise amoebocytes are the only non-germinal cells involved in gametogenesis. In the male, the overlying amoebocytes are always separated from the spermatogenic cells by a basal lamina. In the female, however, large microvilli known as cytospines project from the surface of the oocytes, passing through the basal lamina, and contacting the amoebocytes closely. The precise role of the amoebocytes during gametogenesis is unclear, but they may be involved in gamete nutrition, as has been suggested for amoebocytes in some polychaete annelids (DALES and DIXON 1981). They are also active in phagocytosis in well-developed testicular cysts and during gamete resorption.

It has been suggested that scyphozoan and anthozoan amoebocytes can differentiate into other, possibly more specialized cell types. BUISSON and FRANC (1969) and FRANC (1970) found that the mesenchyme cells of *Veretillium cynomorium* differentiated into nematocytes, nerve cells and mucus-producing cells. CHAPMAN (1974) reports *Aurelia* amoebocytes differentiating into cnidocytes and possibly other cell types. Evidence of this sort led CHAPMAN (1974) to compare and contrast the amoebocytes of *Scyphozoa* and *Anthozoa* with the interstitial cells of *Hydrozoa*. Hydrozoan interstitial cells have been extensively studied, and are known to differentiate into other cell types, forming nerve cells, nematocytes and gametes (for a review, see BODE and DAVID 1978). The situation regarding the occurrence and role of interstitial cells in the *Scyphozoa* and *Anthozoa* is less well understood. They have been reported from among the *Anthozoa*, including from the sea anemones *Metridium senile* (WESTFALL 1968) and *Aiptasia diaphana* (SINGER 1971). Other authors, however, have failed to find interstitial cells or conclude that they are rare or only locally distributed (ROBSON 1957, VAN PRAET 1974, VAN PRAET and DOUMENC 1975). If amoebocytes can differentiate into other cell types, they could, at least in part, perform the role played by the interstitial cells of *Hydrozoa*. In the present study, although the amoebocytes could vary greatly in appearance, no clear evidence of their differentiating into other cell types was obtained. Similarly, YOUNG (1974) could find no evidence of amoebocytes differentiating into other cell types in the sea anemone *Calliactis parasitica*. It is of course possible that such differentiation could take place at low frequency or under particular circumstances in sea anemones, but it is not thought to be a general occurrence.

In spite of the large number of amoebocytes observed during the course of this study, no cells were observed undergoing mitosis. CHAPMAN (1974) examined 400 amoebocytes in *Aurelia* before finding one in mitosis. It has been suggested that mitoses in coelenterates may be rapid (CAMPBELL 1967, DOUMENC 1977) and so rarely observed in histological preparations. Mitosis could also be restricted to localized regions or to brief periods of the day and so easily be missed in studies of this sort. VAN PRAET (1978) has observed groups of cells located in the mesogloea of the upper, trilobed portions of the gastric filaments in *A. equina*, and suggests that they may constitute a reserve of maturing amoebocytes. During the present investigation, these groups of cells in the upper filament were examined in some detail in *A. fragacea* (unpublished observation). They appear to represent a separate cell type, morphologically distinct from the granular amoebocytes, and no evidence that

amoebocytes were derived from these cells was obtained. Autoradiographic analysis of radiolabeled thymidine uptake may be a more reliable guide to cell division in coelenterates than observation of mitotic figures. MINASIAN (1980) investigated the incorporation of tritiated thymidine in the sea anemone *Haliplanella luciae* in this way. He found thymidine incorporation, indicative of cell division, in nuclei of eosinophil granulocytes as well as other cell types. These cells are thought to correspond to the granular amoebocytes described here, and he concluded that they constitute a distinct, renewing population of cells.

It is not possible here to embark upon a comparison between the granular amoebocytes of *A. fragacea* and the wide range of corresponding cells found in other invertebrate groups. They do, however, exhibit strong similarities to several of these, perhaps most notably with those of some polychaetous annelids (see DALES and DIXON 1981). They also display a similar range of functions to polychaete coelomycetes, being motile, phagocytic, and involved in nutrient transport and gametogenesis. Further studies of the origin and functions of the amoebocytes of sea anemones and other lower metazoans may be significant with regard to the evolution of blood cells in other groups.

Acknowledgements

The author is grateful to Dr. M. A. CARTER for helpful discussions and critical reading of a previous draft of this paper, and to Mrs. L. HEALEY for typing the manuscript.

References

BERGQUIST, P. R., 1978: Sponges. London: Hutchinson.

BODE, H. R., DAVID, C. N., 1978: Regulation of a multipotent stem cell, the interstitial cell of hydra. Prog. biophys. molec. Biol. **33**, 189—206.

BOURY-ESNAULT, N., 1977: A cell type in sponges involved in the metabolism of glycogen. The gray cells. Cell Tiss. Res. **175**, 523—539.

BUISSON, B., FRANC, S., 1969: Structure et ultrastructure des cellules mésenchymateuses intramésogléenes de *Veretillium cynomorium* Pall. (Cnidaire, Pennatulidae). Vie Milieu Ser. A **20**, 279—292.

CAMPBELL, R. D., 1967: Tissue dynamics of steady state growth in *Hydra littoralis*. 1. Patterns of cell division. Develop. Biol. **15**, 487—502.

CARTER, M. A., THORPE, J. P., 1981: Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific. J. mar. biol. Ass. U.K. **61**, 79—93.

CHAPMAN, D. M., 1974: Cnidarian histology. In: Coelenterate biology. Reviews and new perspectives (MUSCATINE, L., LENHOFF, H. M., eds.), pp. 2—92. New York: Academic Press.

CHAPMAN, G., 1953: Studies of the mesogloea of *Coelenterates*. 1. Histology and chemical properties. Quart. J. microscop. Sci. **94**, 155—176.

DALES, R. P., DIXON, L. R. J., 1981: Polychaetes. In: Invertebrate blood cells, Vol. 1 (RATCLIFFE, N. A., ROWLEY, A. F., eds.), pp. 35—74. London: Academic Press.

DOUMENC, D. A., 1977: Etude dynamique de la morphogénèse des phases Actinella et Edwardsia de l'actinie *Cereus pedunculatus* Pennant. Arch. Zool. Exp. Gén. **118**, 79—102.

FRANC, S., 1970: Les évolutions cellulaires au cours de la régénération du pédoncule de *Veretillium cynomorium* Pall. Vie Milieu Ser. A **21**, 49—93.

GARRONE, R., POTTU, J., 1973: Collagen biosynthesis in sponges: elaboration of spongin by spongocytes. J. submicrosc. Cytol. **5**, 199—218.

GRIMSTONE, A. V., HORNE, R. W., PANTIN, C. F. A., ROBSON, E. A., 1958: The fine structure of the mesenteries of the sea anemone *Metridium senile*. Quart. J. microscop. Sci. **99**, 523—540.

HYMAN, L. H., 1940: The invertebrates: *Protozoa* through *Ctenophora*. The acoelomate *Bilateria*. New York: McGraw-Hill.

JOHNSTON, M. A., ELDER, H. Y., SPENCER DAVIES, P., 1973: Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism. Comp. Biochem. Physiol. **46 A**, 569—581.

KANESHIRO, E. S., KARP, R. D., 1980: The ultrastructure of coelomocytes of the sea star *Dermasterias imbricata*. Biol. Bull. **159**, 295—310.

KESSEL, R. G., 1968: Electron microscope studies on developing oocytes of a coelenterate medusa with special reference to vitellogenesis. J. Morphol. **126**, 211—248.

LARKMAN, A. U., 1980: Ultrastructural aspects of gametogenesis in *Actinia equina* L. In: Developmental and cellular biology of coelenterates (TARDENT, P., TARDENT, R., eds.), pp. 61—66. Amsterdam: Elsevier/North Holland Biomedical Press.

— 1981: An ultrastructural investigation of the early stages of oocyte differentiation in *Actinia fragacea* (*Cnidaria: Anthozoa*). Int. J. Invertebr. Reprod. **4**, 147—167.

— 1983: An ultrastructural study of oocyte growth within the endoderm and entry into the mesoglea in *Actinia fragacea* (*Cnidaria: Anthozoa*). J. Morphol. **178**, 155—177.

— CARTER, M. A., 1980: The spermatozoon of *Actinia equina* L. var. *mesembryanthemum*. J. mar. biol. Ass. U.K. **60**, 193—204.

— — 1982: Preliminary ultrastructural and autoradiographic evidence that the trophonema of the sea anemone *Actinia fragacea* has a nutritive function. Int. J. Invertebr. Reprod. **4**, 375—379.

LEWIS, P. R., KNIGHT, D. P., 1977: Staining methods for sectioned material. Amsterdam: North Holland Publishing Co.

MINASIAN, L. L., 1980: The distribution of proliferating cells in an anthozoan polyp, *Haliplanella luciae* (*Actinaria: Acontiaria*), as indicated by ^{3}H-thymidine incorporation. In: Developmental and cellular biology of coelenterates (TARDENT, P., TARDENT, R., eds.), pp. 415—420. Amsterdam: Elsevier/North Holland Biomedical Press.

PATTERSON, M. J., LANDOLT, M. L., 1979: Cellular reaction to injury in the anthozoan *Anthopleura elegantissima*. J. Invert. Pathol. **33**, 189—196.

POLTEVA, D. G., 1970: Morphogenetic process in somatic embryogenesis of *Metridium senile*. Vestn. Leningrad Univ. Ser. Biol. **25**, 96—105; Biol. Abstr. **51**, 128—150.

Protoplasma 122, 222–232 (1984)

PROTOPLASMA

Changes in Density of Chromatin in the Meristematic Cells of *Sinapis alba* During Transition to Flowering

A. HAVELANGE [1,*] and J. C. JEANNY [2]

[1] Laboratoire de Physiologie végétale, Département de Botanique, Université de Liège
[2] Laboratoire de Biologie cellulaire, Faculté des Sciences de Reims

Received October 14, 1983
Accepted December 19, 1983

Summary

Changes in the density of nuclear chromatin in the shoot apical meristem of *Sinapis alba* L. during floral transition (floral evocation) are described using Feulgen-stained 2 µm thick semi-thin sections and scanning cytophotometric techniques. In both G 1 and G 2 nuclei the chromatin becomes less heterogeneous and less dense in evoked meristems compared to vegetative meristems. When chromatin is resolved into two fractions the "dispersed" fraction increases relative to the "condensed" fraction at evocation. This decondensation process occurs earlier in G 1 than in G 2 nuclei. These chromatin changes are presumably closely related to the dramatic stimulation of biosynthetic activity and cell division during floral transition.

Keywords: Chromatin; Floral evocation; Shoot meristem; *Sinapis*.

1. Introduction

A variety of studies (reviewed in BERNIER, KINET and SACHS 1981) have shown that the shoot apical meristem of many different plant species is dramatically activated during its transformation from the vegetative to the reproductive condition. This stimulation is well documented in the long-day plant *Sinapis alba* where it includes increases in RNA synthesis and content (BRONCHART *et al.* 1970, JACQMARD *et al.* 1972, PRYKE and BERNIER 1978), DNA synthesis and cell division (BERNIER *et al.* 1967, BODSON 1975) and changes in the protein complement (PIÉRARD *et al.* 1980). Parallel ultrastructural changes were quantitatively investigated by HAVELANGE and BERNIER (1974) using stereological methods. Within the nuclei of meristematic cells, the floral transition is accompanied mainly by a) a large increase in nucleolus size and a change in structure of this organelle, and b) an increase in the dispersed: condensed chromatin area ratio.

This last change was of interest since it is known from studies with animal cells that chromatin activation, *i.e.*, increased chromatin transcription rate, is generally associated with decondensation of this nuclear component (HARRIS 1970, FRENSTER 1974, MACLEAN and HILDER 1977, NAGL 1979). However, before assuming that a similar correlation exists in the meristem of *Sinapis* it is necessary to reinvestigate the problem using techniques that can accurately monitor changes in chromatin condensation. A change in the area of dispersed and condensed chromatins in a nuclear section does not necessarily indicate chromatin decondensation. Also, an accurate distinction between the two types of chromatin is sometimes difficult in electron micrographs. The technique selected for the present study is scanning cytophotometry of semi-thin meristem sections, Feulgen-stained for DNA, the major component of chromatin. Thus, extinction values were measured at multiple points along a series of scanning lines within individual nuclear sections. Two µm thick meristem sections were used to avoid as much as possible superimposition of several chromatin masses of various condensation degrees.

* Correspondence and Reprints: Laboratoire de Physiologie végétale, Département de Botanique, Université de Liège, Sart Tilman, Bât. B 22, B-4000 Liège, Belgium.

PROCKOP, D. J., KIVIRIKKO, K. I., TUDERMAN, L., GUZMAN, N., 1979: The biosynthesis of collagen and its disorders. N. Engl. J. Med. 301, 77—85.

ROBSON, E. A., 1957: The structure and hydromechanics of the musculoepithelium in *Metridium*. Quart. J. Microscop. Sci. 98, 265—270.

SINGER, I. I., 1971: Tentacular and oral-disc regeneration in the sea anemone, *Aiptasia diaphana*. III. Autoradiographic analysis of patterns of tritiated thymidine uptake. J. Embryol. exp. Morph. 26, 253—270.

— 1974: An electron microscopic and autoradiographic study of mesogloeal organization and collagen synthesis in the sea anemone *Aiptasia diaphana*. Cell Tiss. Res. 149, 537—554.

SPANGENBERG, D. B., BECK, C. W., 1968: Calcium sulfate dihydrate statoliths in *Aurelia*. Trans. Amer. microsc. Soc. 87, 329—335.

TARDENT, P., SCHMID, 1973: Ultrastructure of mechanoreceptors of the polyp *Coryne pintneri* (*Hydrozoa, Athecata*). Exp. Cell Res. 72, 265—275.

— TARDENT, R., 1980: Developmental and cellular biology of coelenterates. Amsterdam: Elsevier/North Holland Biomedical Press.

TIFFON, T., HUGON, J. S., 1977: Localisation ultrastructurale de la phosphatase acide et de la phosphatase alcaline dans les cloisons septales stériles de l'anthozoaire *Pachycerianthus fimbriatus*. Histochemistry 54, 289—297.

VAN DER VYVER, G., 1981: Organisms without special circulatory systems. In: Invertebrate blood cells, Vol. 1 (RATCLIFFE, N. A., ROWLEY, A. F., eds.), pp. 19—32. London: Academic Press.

VAN PRAET, M., 1974: Régénération de la région péri-orale d'*Actinia equina* L. Thèse 3e cycle, Université Paris VI.

— 1976: Les activités phosphatasiques acides chez *Actinia equina* L. et *Cereus pedunculatus* P. Bull. Soc. Zool. France 101, 367—376.

— 1978: Etude histochimique et ultrastructurale des zones digestives d'*Actinia equina* L. (*Cnidaria, Actinaria*). Cah. Biol. Mar. 19, 415—432.

VAN PRAET, M., DOUMENC, D., 1975: Morphologie et morphogénèse expérimentale du tentacule chez *Actinia equina* L. J. Microsc. Biol. Cell 23, 29—38.

WATSON, G. M., MARISCAL, R. N., 1983: Comparative ultrastructure of catch tentacles and feeding tentacles in the sea anemone *Haliplanella*. Tiss. Cell 15, 939—953.

WESTFALL, J. A., 1966: The differentiation of nematocysts and associated structures in the *Cnidaria*. Z. Zellforsch. 75, 381—403.

YOUNG, J. A. C., 1974: The nature of tissue regeneration after wounding in the sea anemone *Calliactis parasitica* (Couch). J. mar. biol. Ass. U.K. 54, 599—617.

Printed in Austria